Effect of xanthine oxidase-generated extracellular superoxide on skeletal muscle force generation.

Gomez-Cabrera MC\textsuperscript{1}, Close GL\textsuperscript{2}, Kayani A\textsuperscript{2}, McArdle A\textsuperscript{2}, Viña J\textsuperscript{1} and Jackson MJ\textsuperscript{2}

\textsuperscript{1}Department of Physiology, Faculty of Medicine, University of Valencia, Valencia, Spain
\textsuperscript{2}Pathophysiology Research Unit, School of Clinical Sciences, University of Liverpool, Liverpool, UK.

Running title: Xanthine oxidase-derived free radicals in skeletal muscle

Correspondence to:
Professor Malcolm J Jackson.
Tel : 01517064074
Fax: 01517065802
e-mail: m.j.jackson@liverpool.ac.uk

Copyright © 2009 by the American Physiological Society.
Abstract

Skeletal muscle contractions increase superoxide anion in skeletal muscle extracellular space. We tested the hypotheses that, (i) after an isometric contraction protocol, xanthine oxidase (XO) activity is a source of superoxide anion in the extracellular space of skeletal muscle, and (ii) the increase in XO-derived extracellular superoxide anion during contractions affects skeletal muscle contractile function. Superoxide anion was monitored in the extracellular space of mouse gastrocnemius muscles by following the reduction of cytochrome c in muscle microdialysates. A 15-min protocol of non-damaging isometric contractions increased the reduction of cytochrome c in microdialysates indicating an increase in superoxide anion. Mice treated with the xanthine oxidase inhibitor, oxypurinol, showed a smaller increase in superoxide anions in muscle microdialysates following contractions than was seen in microdialysates from muscles of vehicle-treated mice. Intact extensor digitorum longus (EDL) and soleus muscles from mice were also incubated in vitro with oxypurinol or polyethylene glycol-tagged superoxide dismutase (PEG-SOD). Oxypurinol treatment decreased the maximum tetanic force produced by both EDL and soleus muscles and PEG-SOD treatment decreased the maximum force production by the EDL muscles. Neither agent influenced the rate of decline in force production when EDL or soleus muscles were repeatedly electrically stimulated using a 5 minute fatiguing protocol (stimulation at 40Hz for 0.1 second every 5 seconds). Thus these studies indicate that XO activity contributes to the increased superoxide anion detected within the extracellular space of skeletal muscles during non-damaging contractile activity and that XO-derived superoxide anion or derivatives of this radical have a positive effect on muscle force generation during isometric contractions of mouse skeletal muscles.

Key words: Contractile function, Free radicals, Exercise
Introduction

In 1954, Commoner and colleagues reported that skeletal muscle contains free radical species (10) but the biological importance of this finding was unclear until the early 1980s when researchers identified a potential link between muscle function and free radical generation (12, 24). Further studies have shown that, in some circumstances, contractile activity of muscle can lead to altered muscle and blood glutathione levels and an increase in both protein and DNA oxidation (41, 47). The free radicals produced from skeletal muscle appear to be involved in a number of physiological processes, including excitation-contraction coupling (43) and cell signalling (23). ROS can also activate redox-sensitive transcription factors (20, 25). This activation can lead to increased expression of regulatory enzymes such as superoxide dismutase (SOD), cytoprotective proteins such as heat shock proteins (HSPs) (28, 34) and other enzymes, such as iNOS and eNOS (14).

In order to understand the role that ROS play in skeletal muscle, it is essential to identify and quantify specific ROS and establish their sites of production in muscle (22). During and after contractile activity, ROS production may be increased from several cellular sites such as the mitochondrial respiratory chain, NADPH oxidases and activated phagocytes (22, 25) and also potentially from xanthine oxidase enzymes. Xanthine oxidase ( XO) and xanthine dehydrogenase (XDH) are isoenzymes of xanthine oxidoreductase, which catalyze the oxidation of hypoxanthine and xanthine to urate during purine catabolism in mammals. While XDH preferentially transfers the electrons released during the oxidation process to NAD+, XO utilizes molecular oxygen, thereby generating superoxide anion (17). Treatment with allopurinol, or its active metabolite, oxypurinol that blocks XO activity by binding at its active site (55), have been associated with a decrease in the levels of both indicators of oxidative damage and markers of muscle damage after exhaustive exercise protocols in both humans and rats (14, 16).

Detection of ROS in biological systems is difficult, since these species occur at very low concentrations and react rapidly with cellular components close to their sites of formation, thus having little capacity to accumulate. Since the primary ROS generated by skeletal muscle (superoxide and nitric oxide) are found close to their site of synthesis, an assay system that is designed to measure specific primary ROS must have access to this site. One technique that permits this in the interstitial space of tissues is microdialysis (37). We have previously used this technique to monitor the extracellular activity of superoxide anion in muscle interstitial space during a protocol of isometric contractile activity (9, 34).
The aim of the current study was to examine the role of XO activity in the contraction-induced increase in superoxide anion detected in the extracellular fluid of mouse skeletal muscle. Having established that XO activity contributes to the contraction-induced increase in extracellular superoxide anion, further studies were undertaken to determine the effects of inhibition of superoxide generation by XO or scavenging of extracellular superoxide on the contractile properties of skeletal muscle.
Methods

In vivo studies

Mice and drug administration
In the first series of experiments adult (3 month old, 30g weight) male C57BL/6 mice were randomly divided into three experimental groups that were given an intravenous injection of 0.2 ml saline (n=12), 0.2 ml vehicle (n=10) or 0.2 ml oxypurinol (0.67mM, n=12). The vehicle for oxypurinol contained 25mM NaOH, 92.5mM NaHCO$_3$ at pH 7.4 (39). Mice were anaesthetised with pentobarbitone sodium (7.3mg/100g i.p.). Supplemental doses of anaesthetic were administered as required to maintain deep anaesthesia such that mice were not responsive to tactile stimuli throughout the procedure. The drug treatments were administered by intravenous tail vein injection when the mice were fully anaesthetised. Thirty minutes following induction of anaesthesia, a microdialysis probe (MAB 3.35.4, Metalant, Stockholm, Sweden), with a molecular mass cutoff of 35 kDa, was placed into the gastrocnemius muscle of the right hind limb.

All experiments were performed in accordance with UK Home Office Guidelines under the UK Animals (Scientific Procedures) Act 1986.

Microdialysis studies
The microdialysis probes were perfused with 50 μM cytochrome c in saline at a flow rate of 4 μl/min (9). Microdialysates were collected every 15 min resulting in a total of 60 μl of dialysate per collection. Following four 15 minute baseline collections, the right hind limb of the anesthetised mouse was subjected to a 15-min period of isometric contractions by electrical stimulation using surface electrodes placed around the upper limb and ankle as previously described (9, 34). In most experiments the mouse muscles were stimulated for 15 min with square wave pulses of 0.1 ms in duration at 100 Hz and 30V for 0.5s every 5s (34), but in a limited number of experiments the effect of a protocol of lower frequency stimulations was examined (square wave pulses of 0.1 ms in duration at 59 Hz and 30 V for 0.5s every 5s). Following the period of contractions, two further 15 minute collections of microdialysates were undertaken with the muscles at rest. Mice remained under anaesthesia until the end of the experiment and were then killed by overdose of pentobarbitone sodium. Gastrocnemius and liver samples were rapidly dissected, frozen in liquid nitrogen, and stored at -80°C until analyzed.
Measurement of extracellular superoxide
The reduction of cytochrome c was analysed by spectrophotometry as previously described (9, 34). Briefly, cytochrome c samples were diluted 1 in 5 with distilled water and analysed using scanning visible spectroscopy. The reduction of cytochrome c was calculated from the absorbance at 550 nm compared with that at the isosbestic wavelength of 542 nm. Results are expressed as superoxide equivalents using a molar extinction coefficient for reduced cytochrome c of 21,000 (9).

Muscle force-frequency relationship
The force frequency relationship for extensor digitorum longus (EDL) muscles was analysed as previously described (27). Mice were anaesthetised as previously and the knee of the right hindlimb was fixed. The distal tendon of the EDL muscle was exposed and attached to the lever arm of a servomotor (Cambridge Technology, USA). The peroneal nerve was exposed and stainless steel needle electrodes were placed across the nerve. Stimulation voltage and muscle length were adjusted to produce a maximum twitch force. The muscle length that produced the maximum twitch force is also the optimum muscle length (L₀) for the production of maximum tetanic force (P₀). The muscle was electrically stimulated to contract at L₀ and optimal stimulation voltage (8 – 10V) every 2 minutes for a total time of 500ms with 0.2ms pulse width. The frequency of stimulation was increased for each 500ms stimulus from 10Hz to 50Hz and subsequently in 50Hz increments to a maximum of 400Hz.

In vitro studies
In the second series of experiments adult (3 month old) male C57BL/6 mice were killed with an overdose of pentobarbitone sodium and soleus and EDL muscles were rapidly removed from both limbs in random order. The muscles were mounted at constant length in a tissue bath containing oxygenated, mammalian Ringer’s solution: NaCl (137mM), KCl (5mM), CaCl₂ (2mM), MgSO₄, (1mM), NaH₂PO₄ (1mM), NaHCO₃ (24mM), and tubocurarine chloride (0.025mM). The solution was gassed continually with 95% O₂, 5% CO₂ throughout the experiment. Temperature was maintained at 37°C. Muscles were randomly divided into three experimental groups: control with no additions (n=10), incubated with oxypurinol (final concentration 0.67mM, n=10) and incubated with PEG-tagged Cu,Zn superoxide dismutase (PEG-SOD, final activity 500 U/ml, n=10). Oxypurinol and PEG-SOD were obtained from Sigma Chemical Co. Ltd. (Poole, Dorset, UK) and prepared daily in mammalian Ringer’s solution.
**Muscle contractile properties**

*Soleus* and *EDL* muscles were studied in vitro, the preparation and experimental conditions have been described previously (48). Following 1 hour pre-incubation with oxypurinol (14) or 30 minutes pre-incubation with PEG-SOD (44), optimal muscle length ($L_0$) for peak twitch force was established for the isolated muscles. All subsequent measures were made at $L_0$. Muscles were electrically stimulated between two platinum electrodes with 0.1 ms square-wave pulses of supramaximal voltage. Force production was monitored with a force transducer (Linton Instrumentation, UK) coupled to a storage oscilloscope (Gould Type 1425, UK). Contractile characteristics including time to peak twitch (TPT), twitch half relaxation time (RT$_{1/2}$) and peak twitch force ($P_t$) were measured. Stimulation frequency was increased in both *EDL* and *soleus* muscles until maximum isometric tetanic force ($P_0$) was obtained; muscles were rested for 2 min between each of these contractions (48). Prior to a fatigue test, each muscle was rested for 5 min after measurement of $P_0$.

To allow expression of $P_0$ as maximum specific tension (N/cm$^2$), the weight and length of each muscle was measured at the end of each experiment and muscle cross-sectional area was calculated (48).

**Fatigue protocol**

*Soleus* and *EDL* muscles were electrically stimulated for 5 min with square wave pulses of 0.1 ms in duration at 40 Hz and 60 V for 0.1 s every 5 s. The force generated was measured throughout the fatiguing protocol. Two minutes after the end of the protocol of repeated stimulations, the muscles were electrically stimulated with a single stimulus and the force generation was measured again in order to calculate the percentage recovery (26).

**Measurement of Xanthine Oxidase Activity**

XO activity was determined fluorometrically (5). Frozen liver tissue (0.2g/mL) was homogenised in 0.25 M sucrose, 10 mM DTT, 0.2 mM PMSF, 0.1 mM EDTA and 50 mM potassium phosphate, pH 7.4. Homogenates were centrifuged for 30 minutes at 15,000g and activities measured in supernatants. The reaction was initiated by addition of pterin (0.010 mmol/L) as a substrate, XO activity was obtained from the rate of increase in fluorescence due to the conversion of pterin to isoxanthopterin. The reaction was stopped by addition of allopurinol (50 µmol/L). To calibrate the assay, the fluorescence of a standard concentration of isoxanthopterin was measured. Protein concentration of homogenates was determined by the Bradford assay (6).
**Statistical analyses**

Statistical analyses were carried out using the Statistical Package (SPSS version 11.01). All data are presented as means ± SE. A one-way repeated-measures ANOVA was used to analyse the effects of the contraction protocol on the reduction of cytochrome c. A mixed design factorial ANOVA was used to examine the effects of the ROS inhibitors. When Mauchley's test of sphericity indicated a minimum level of violation (Greenhouse Geisser epsilon >0.75) data were corrected using the Huynh-Feldt epsilon; however, when sphericity was deemed to be violated (Greenhouse Geisser epsilon <0.75) data were corrected using the Greenhouse Geisser epsilon. Where a significant F value was observed, Tukey's HSD post hoc analysis was performed to identify where the significant differences occurred. Statistical significance was set at the level of 0.05 for all of the tests.
Results

Superoxide anion in muscle extracellular space. Effect of oxypurinol administration.
The changes in cytochrome c reduction (presented as superoxide equivalents) in microdialysates from resting and contracting muscle from control mice, mice given an IV injection of vehicle and mice treated with oxypurinol are shown in Figure 1. The protocol of 180 isometric contractions induced a significant increase in cytochrome c reduction in microdialysates from the mice given saline or vehicle, but prior injection of mice with oxypurinol prevented this contraction-induced increase in the reduction of cytochrome c. XO activity was determined in the liver tissue of mice, since XO activity is known to be high in this tissue (21). Liver XO activity decreased significantly from 2058.6 (SE 388.3) mU/g protein in the saline and 1926.3 (SE 356.1) mU/g protein in the vehicle groups to 94.3 (SE 36.8) mU/g protein in the oxypurinol treated mice (n=6, p< 0.01).

Effect of treatment with oxypurinol or PEG-SOD on the contractile properties of EDL and soleus muscles
Table 1 shows the effect of incubation of EDL or soleus muscles with oxypurinol or with PEG-SOD on the maximum tetanic force generated by EDL and soleus muscles. Treatment with oxypurinol caused a significant reduction in the maximum force generation by both EDL and soleus muscles. Incubation with PEG-SOD caused a significant reduction in maximum force generation for EDL muscles, but had no effect on soleus force generation. Both oxypurinol and PEG-SOD treatments reduced significantly the peak twitch force generation by the EDL muscle (data not shown in detail).

Effect of treatment with oxypurinol or SOD on fatigue and recovery from fatigue in EDL and soleus muscles.
Figures 2A and 2B show the loss of force generation by EDL and soleus muscles over time during repetitive tetanic contractions at 40 Hz. EDL muscles lost a greater proportion of force over 5 minutes of repeated stimulations than soleus muscles, but the loss of force generation in both EDL or soleus muscles was unaffected by exposure to oxypurinol or PEG-SOD. Similarly neither agent significantly affected the recovery of force at 2 minutes following contractions (data not shown in detail).

Effect of reduced muscle force generation on the contraction-induced generation of superoxide anion.
The data presented in Figure 1 and Table 1 show that exposure of muscle to oxypurinol induced a significant depression in maximum tetanic force generation by both EDL and
soleus muscles and a reduction in contraction-induced extracellular superoxide anion. It appeared feasible that the effect of oxyipurinol to reduce muscle force generation may have been responsible for the reduced superoxide release, rather than any direct effect of oxyipurinol on xanthine oxidase activity reducing superoxide release. An experiment was undertaken to examine this possibility in which untreated gastrocnemius muscles were electrically stimulated using a modified contraction protocol in which the stimulation frequency was decreased to produce an equivalent force to that seen in the oxyipurinol-treated muscles was calculated from the force frequency curve for EDL muscles (Figure 3A). A reduction in stimulation frequency to 59Hz was calculated to produce an equivalent force generation to that achieved at 100Hz in muscles from oxyipurinol treated mice. The data in Figure 3B shows that the increase in extracellular superoxide anion was unaffected by the frequency of stimulation and hence force generation.
Discussion

**The role of XO in superoxide generation during muscle contraction.**

Hellsten et al. (18) first showed that XO is an important source of ROS generation during exercise and demonstrated that a chronic exercise protocol increased XO activity in human muscle. The enzyme was found to be present mainly in microvascular endothelial cells and in infiltrating leucocytes (19). It was subsequently demonstrated that rats exercised to exhaustion had higher plasma XO activities than control non-exercised rats (14) and a linear correlation between plasma XO activity and lactate concentration in exercised rats has been described (40). Inhibition of XO using allopurinol was also found to reduce markers of muscle damage associated with exhaustive exercise in rats (56) and humans (16) and XO-derived ROS were shown to be important in activating signalling pathways involved in muscle adaptations to exercise (14, 25).

In the current study the role of XO in superoxide generation in the extracellular space of skeletal muscle *in vivo* has been examined. The effect of a non-damaging contraction protocol on the reduction of cytochrome c in microdialysates obtained from the gastrocnemius muscle extracellular space has been studied as a measure of net superoxide activity during a contraction protocol. The specificity of this assay for superoxide in muscle dialysates has been confirmed by Close et al. (9). Figure 1 shows that intravenous injection of oxypurinol prevented the contraction-induced increase in the reduction of cytochrome c in skeletal muscle microdialysates. Our results are in accord with data from Stofan et al (52) who reported a partial reduction in superoxide anion release from the contracting diaphragm treated with oxypurinol. In addition Matuszczak and colleagues (33) also gave allopurinol to mice and showed a reduction in ROS formation in the cytosol of soleus muscles. In addition to the ability to inhibit xanthine oxidase, allopurinol and oxypurinol have weak hydroxyl radical scavenging properties at higher concentrations than those used here (36). Hence our data support the theory that XO is involved in superoxide radical release into the extracellular fluid during a non-damaging protocol of muscle contractions.

Release of superoxide from cultured muscle cells has been reported (34), but the data presented here suggest that the superoxide responsible for extracellular cytochrome c reduction may also be derived from XO activity in the endothelium of skeletal muscle. It has been suggested that muscle contraction alters the shear stresses applied to the vascular bed of the muscle and that this latter stimulus induces superoxide formation and release (52). Multiple free radical-generating pathways have been reported in skeletal
muscle (22) and the present data argue that the XO pathway is important in superoxide formation in the extracellular fluid following a non-damaging protocol of muscle contractions in addition to the acknowledged activity of XO during and following tissue ischaemia.

Reduction of superoxide anion in the extracellular space and muscle force generation in non-fatigued muscles

Contractile activity of skeletal muscle may lead to oxidation of many biomolecules indicated by altered muscle and blood glutathione levels and an increase in protein, DNA and lipid oxidation (41, 47). Several studies have examined whether increasing the intracellular levels of antioxidants within a muscle cell can provide protection against oxidation and reduce muscle fatigue (49), but Reid and co-workers (44) also demonstrated that in non-fatigued skeletal muscle, ROS appear to have a positive effect on excitation-contraction coupling and are obligatory for optimal contractile function. Specifically they demonstrated that addition of catalase and SOD resulted in a diminished in vitro muscle contractile performance in unfatigued muscle (44). Furthermore addition of strong synthetic reducing agents or antioxidants such as dithiothreitol (2), N-acetylcysteine (29), and DMSO (45, 46) to skeletal muscle in an organ bath resulted in a reduction in skeletal muscle force production. These data have been supported by other studies where exposure to a ROS-generating system (XO and hypoxanthine) or hydrogen peroxide resulted in an increase in low frequency-stimulated force generation by the non-fatigued diaphragm (44). The data in Table 1 show that prevention of superoxide generation by inhibition of XO activity depressed skeletal muscle force production in both EDL and soleus muscles. Maximum tetanic force generation by EDL muscles was depressed by ~57% and in soleus muscle by ~38% following incubation with oxypurinol. Incubation of the muscles with PEG-SOD (Table 1) caused a 34% decrease in tetanic force generation by EDL muscles but had no effect on force generation by soleus muscles. While these data may suggest that PEG-SOD is more effective in preventing superoxide induced effects on muscle force production in type II fibers, they may also reflect an inability of the added PEG-SOD to compete for superoxide in the presence of other potential reactants such as nitric oxide, the concentrations of which may vary between different muscle types. Other workers have studied the effect of antioxidants on force generation by skeletal muscle and have demonstrated a reduction in sub-maximal force generation of non-fatigued muscle at low frequencies of stimulation, but no similar effects on maximal tetanic force generation has been reported except where very high (and potentially toxic) concentrations of antioxidants were used (e.g. see 8, 29, 42, 44). It is not immediately clear why the data reported here differ from those previously reported, but the published
data are derived from multiple models and none are identical to the model studied here. In particular there are differences in the muscle type studied (diaphragm, gastrocnemius, EDL or soleus muscles), the species studied (rats, mice or dogs), the nature and concentration of the antioxidant used, and the temperature and incubation periods used. Thus a variety of factors may have contributed to the different effects observed.

One aspect that may be important is the temperature and the duration of incubation period. Thus in the current study mouse muscle was studied at 37°C whereas some previous data were obtained at lower temperatures. Muscle-derived ROS activities have been reported to be diminished at room temperature (3) and antioxidants that inhibit fatigue at 37°C have been reported to have no effect at 23°C (13). Our experimental model utilised an extended duration of exposure of the muscle to antioxidants at 37°C in comparison with most other studies.

Variations in the concentrations of antioxidant used might also explain some of the differences observed in our study. There is no consensus of the concentration of oxypurinol necessary to inhibit xanthine oxidase in skeletal muscle but our protocol differs from that used by other authors. In the current experiments the muscles were incubated with 0.67mM oxypurinol, a concentration which we had observed previously inhibited xanthine oxidase activity in rats (39). We also determined the xanthine oxidase activity in the current study and found a significant decrease after this protocol of administration. In comparison Stofan et al. (52) injected rats intraperitoneally with oxypurinol (50 mg/Kg) at 12 h prior to the study and an additional dose of oxypurinol (50mg/L) was also added to the diaphragmatic perfusate used during the in vitro experiments. These authors did not measure the xanthine oxidase activity. Supinski and colleagues (54) examined fatigue and free radical-mediated lipid peroxidation in the rat diaphragm during loaded breathing by administering oxypurinol and showed no effect of oxypurinol on maximal diaphragm force generation in an in situ diaphragm preparation. Oxypurinol (50 mg/kg) was again administered on the day before the study and an additional 50 mg/kg was given after 30 min of equilibration of the in situ model. The effects of allopurinol were also studied by Barclay and Hansel (4) who examined mouse soleus and canine gastrocnemius-plantaris preparations using in vitro and in situ models. In the canine experiments, the authors determined the effect of 1mM allopurinol on the fatigue rate of blood perfused canine gastrocnemius in situ but saw no significant effect of allopurinol. In a later study, Matuszczak et al. gave allopurinol to mice (50mg/Kg/day) and also observed no loss of maximum force generation by the soleus muscle (33). For studies of PEG-SOD, we examined the effect of 500U/L on isolated muscles whereas Callahan and colleagues (8)
gave PEG-SOD by intraperitoneal injection at a dose of 2000 U/kg to rats and also incubated skinned muscle fibers isolated from the diaphragm with 2000 U/L PEG-SOD. Callaghan et al (8) did not observe any effect on maximum tetanic force generation with either treatment protocol.

The loss of contractile function that was observed following exposure to oxypurinol in electrically stimulated EDL and soleus muscles or PEG-SOD in EDL muscles suggests that these agents alter processes within the intact muscle cell. In this study any intracellular effect of the SOD is likely to have been indirect because the molecular mass of PEG-SOD restricts it to extracellular distribution. XO is located in the skeletal muscle endothelium. Reid and colleagues examined how SOD and catalase added to the extracellular medium might influence intracellular processes mediated by ROS (44). They proposed that the enzyme substrates, hydrogen peroxide and superoxide anions, can cross cell membranes and hence their rapid removal by exogenous enzymes would establish an extracellular “sink” for these endogenous oxidants, maintaining a gradient for diffusion from the cytosol and preventing diffusion back into the cell. In support of this postulate the current study has shown that non-fatigued muscles release superoxide to the extracellular fluid and that incubation with oxypurinol or PEG-SOD (9) lowered the extracellular superoxide. Other research groups have previously shown that incubation of non-fatigued muscles with both SOD or catalase lowered the activities of ROS measured in the muscle cytosol by a non-specific indicator (42).

The mechanisms involved in the modulation of force generation by ROS are not fully established, but published data indicate that the variation of force in response to shifts in the redox balance may be mediated by changes in myofibrillar calcium sensitivity (2) and/or by a reduction in calcium release from the sarcoplasmic reticulum (1, 57). The proteins that determine the calcium sensitivity of the contractile process are troponin and the regulatory myosin light chain (7, 35). It is also possible that superoxide generated during contractile activity acts to decrease nitric oxide (NO) bioavailability in untreated muscles and hence when superoxide generation is inhibited by oxypurinol or SOD, NO bioavailability is increased with beneficial effects on contractile function. To test this possibility, we determined the NO levels in muscle extracellular space during the contractile protocol using the microdialysis technique, but treatment with oxypurinol had no effect on microdialysate NO levels (data not shown in detail).

**Reduction of superoxide anion in the extracellular space and force generation in muscles subjected to repeated stimulation of contractions**
Muscle contractions increase the production of ROS and it has been suggested that the increased generation of these species influences the intracellular redox state to induce a more oxidising environment. Furthermore these perturbations may result in oxidative modifications in contractile proteins that depress contractile function. In previous studies SOD and catalase and other agents that reduce ROS (NAC, DMSO, DTT, allopurinol and desferoxamine) have been reported to slow the loss of contractile force that occurs during repeated electrical stimulation of contractions in muscle preparations in vitro and in situ (4, 49, 53). These findings have not been universally observed, Shrier et al. (50) reported no effect of SOD, polyethylene glycol-conjugated catalase, or desferoxamine on skeletal muscle fatigue. In order to explain these contradictory results, Reid et al. (42) proposed that the effects of ROS scavengers depend on the fatigue protocol used and that fatigue is delayed by these agents during contractile protocols using sub-maximal activation patterns but not when contraction is at maximal or near-maximal intensities (42). These authors suggested that low frequency fatigue in vitro mimics the metabolic changes produced by peripheral fatigue in vivo: glycogen stores and phosphocreatine levels are depleted, intracellular pH falls, and Pi levels rise. Acidosis and increased Pi each inhibit actin-myosin interaction, precipitating contractile failure and that this type of fatigue is also associated with oxidative stress (51). In contrast, high-frequency fatigue was attributed to failure of impulse propagation across the sarcolemma; force declines rapidly under these conditions and recovers within seconds-to-minutes after the end of contractions. Previous work indicates that this latter form of fatigue is not mediated by ROS intermediates (42). Our data show that prevention of the contraction-induced formation of superoxide anions in the muscle extracellular space with the administration of oxypurinol or scavenging of the extracellular superoxide with PEG-SOD did not prevent the loss of muscle force generation that occurs with repeated stimulation at 40Hz for 0.1 sec every 5 sec.

We also examined the possibility that the decreased generation of force by muscle following treatment with oxypurinol might account for the decrease in extracellular superoxide anion produced by the contracting muscle. Control muscles were electrically stimulated with a decreased stimulation frequency that produced an equivalent force generation to that seen in the oxypurinol-treated group, but no differences in the stimulation-induced increase in cytochrome c reduction were seen between the two stimulation protocols (Figure 3). Some previous data also indicate that the release of superoxide from muscle cells is activated by contractions, but not directly related to the frequency of stimulation (38) and the current data are in general agreement with this.

Persepectives and Significance
We conclude that xanthine oxidase is a source of the elevated superoxide anion detected in skeletal muscle extracellular space during non-damaging contractions since intravenous administration of oxypurinol prevented the contraction-induced increase in cytochrome c reduction in microdialysates from the gastrocnemius muscle. Selective inhibition of XO-induced superoxide generation by treatment with oxypurinol also caused a significant decrease in the maximum tetanic force generated by EDL or soleus muscles. Similar data were obtained following treatment of EDL muscles with PEG-SOD. Despite the decreased force generation by non-fatigued, oxypurinol-treated muscles compared with untreated controls, neither pretreatment with PEG-SOD nor oxypurinol delayed the development of fatigue in soleus and EDL muscles subjected to repeated stimulation over 5 minutes.

There has been considerable debate about whether xanthine dehydrogenase and/or oxidase enzymes are present in human muscle to the same extent as they are found in rodent tissue, but the enzymes are acknowledged to be present in endothelial cells from both humans and rodent models (e.g. see (18, 19)). The efficacy of allopurinol against muscle damage in some models of exercise-induced muscle damage in humans (16) indicates that even if the enzyme location is limited to endothelial cells in man, they are able to influence human muscle function. A number of studies have recently suggested that dietary supplementation with agents that prevent ROS formation or scavenge these species does not improve, or may reduce, exercise performance (11, 15, 30-32) and the data presented here indicating a positive effect of XO-derived superoxide anions on muscle force generation are in agreement with these studies.

Grants
The authors would like to thank the Wellcome Trust (Grant number 073263/Z/03) for financial support.
REFERENCES


Table 1. Effect of oxypurinol and PEG-SOD on the maximum tetanic force generated by EDL and soleus muscles.

<table>
<thead>
<tr>
<th></th>
<th>Oxypurinol treated</th>
<th>PEG-SOD treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Maximum tetanic force, $P_0$ (mN)</td>
<td>Maximum tetanic force, $P_0$ (mN)</td>
</tr>
<tr>
<td></td>
<td>Control$^1$</td>
<td>Oxypurinol treated</td>
</tr>
<tr>
<td><strong>EDL</strong></td>
<td>381.0 ± 51.0 (n=9)</td>
<td>241.8 ± 28.5* (n=9)</td>
</tr>
<tr>
<td><strong>Soleus</strong></td>
<td>247.0 ± 39.0 (n=9)</td>
<td>163.2 ± 19.2* (n=9)</td>
</tr>
</tbody>
</table>

Values are presented as means ± SE for the number of muscles in parentheses. *p<0.05 versus appropriate control. $^1$ Control muscles were incubated in mammalian Ringer’s solution for an equivalent time to that of the appropriate test muscles (30 min for PEG-SOD treated muscles and 60 min for oxypurinol-treated muscles), prior to measurement of the maximal tetanic force production.
LEGENDS TO FIGURES

**Figure 1.** Reduction of cytochrome c in microdialysates from *gastrocnemius* muscle of control, saline-treated mice (grey bars), mice treated with vehicle (open bars) or mice treated with oxypurinol (black bars). Muscles were stimulated to contract during the 15-minute period indicated. Data are presented as mean ± SE and expressed as superoxide equivalents. Saline, n=12; vehicle, n=10; oxypurinol, n=12. *p<0.05 versus saline at the same time point. # p<0.05 versus vehicle at the same time point.

**Figure 2.**
**Figure 2A.** Force production by *EDL* muscles during a 5 minute fatigue protocol. Data from control muscles incubated in mammalian Ringer’s solution alone (open symbols), oxypurinol (black symbols) and PEG-SOD (grey symbols) are shown as a percentage of the initial force generated. Data are presented as mean ± SE. Control, n=9; Oxypurinol-treated, n=9; PEG-SOD-treated, n=10. No significant differences were seen between the different groups of muscles.

**Figure 2B.** Force production by *soleus* muscles during a 5 minute fatigue protocol. Data from control muscles incubated in mammalian Ringer’s solution alone (open symbols), oxypurinol (black symbols) and PEG-SOD (grey symbols) are shown as a percentage of the initial force generated. Data are presented as mean ± SE. Control, n=9; Oxypurinol-treated, n=9; PEG-SOD-treated, n=10. No significant differences were seen between the different groups of muscles.

**Figure 3.**
**Figure 3A.** Force frequency relationship for mouse *EDL* muscles

**Figure 3B.** Reduction of cytochrome c in microdialysates from *gastrocnemius* muscle of control mice. Muscles were stimulated to contract during the 15-minute period indicated with a protocol of electrical stimuli at 100Hz (black bars) or 59Hz (open bars). Data are presented as mean ± SE and expressed as superoxide equivalents. 100Hz, n=4; 59Hz, n=4. * p< 0.05 compared with values obtained immediately prior to contractile activity (45–60 min). # p< 0.05 compared with values obtained immediately prior to contractile activity (45–60 min). No differences were seen between the reduction in cytochrome c with the two different stimulation protocols.
Figure 1.

The figure shows the superoxide equivalents (nmole/15 min) over time (minutes) during different phases: Baseline, Stimulation, and Recovery. The y-axis represents superoxide equivalents, while the x-axis shows time in minutes, divided into intervals: 0-15, 15-30, 30-45, 45-60, 60-75, 75-90, 90-105.

During the Baseline phase, the superoxide equivalents are relatively constant across all time intervals. During the Stimulation phase, there is a significant increase in superoxide equivalents, indicated by the asterisk (*) mark. The Recovery phase shows a return to baseline levels, with the number sign (#) indicating a statistically significant difference compared to the Baseline period.
Figure 2.
Figure 3.