J Musculoskelet Neuronal Interact 2010; 10(4):267-273

Original Article

Hylonome

Effects of oxidation on the power of chemically skinned rat soleus fibres

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Abstract

Oxidation alters calcium sensitivity, and decreases maximum isometric force (*Po*) and shortening velocity (*Vmax*) of single muscle fibres. To examine the effect of oxidation on the curvature of the force-velocity relationship, which determines muscle power in addition to *Po* and *Vmax*, skinned rat type I fibres were maximally activated at 15ºC in a solution with pCa 4.5 and subjected to isotonic contractions before and after 4-min incubation in 50 mM H_2O_2 (n=10) or normal relaxing solution (n=3). In five oxidised and four control fibres the rate of force redevelopment (*ktr*), following a rapid release and re-stretch, was measured. This gives a measure of the sum of the rate constants for cross-bridge attachment (f) and detachment (g_1): (f+g_1). H_2O_2 reduced *Po*, *Vmax* and *ktr* by 19%, 21% and 24% respectively (P<0.001), while the shape of the force-velocity relationship was unchanged. Fitting data to the Huxley cross-bridge model suggested that oxidation decreased both the rate constant for cross-bridge attachment (f), and detachment of negatively strained cross-bridges (g_2), similar to the effect of reduced activation[1]. This suggests that oxidative modification is a possible cause of the variation in contractile properties between muscle fibres of the same type[2].

Keywords: Skeletal Muscle, Skinned Fibres, Power, Force-Velocity Relationship, Oxidation

Introduction

There are several reasons why it is of interest to study the effect of oxidative modification on the contractile characteristics of single muscle fibres. Ageing, for instance, is associated with deterioration in muscle function that has been suggested to be a consequence of oxidative damage[3,4]. From a mechanistic viewpoint, oxidative modification may alter specific components of the contractile apparatus and thus throw light on the factors that determine the characteristic shape of the force-velocity relationship. Oxidative modification of proteins in the myofilament lattice may cause the wide variation in specific power[5], as a consequence of differences in maximal shortening velocity (*Vmax*), specific tension (*Po*) and curvature of the force-velocity relationship (indicated by *a*/*Po* in the Hill equation where higher values of *a*/*Po* indicate less curvature[6]), even in fibres of the same myosin heavy chain (MHC) type[2].

It has been shown that in skinned fibres the curvature of the force-velocity relationship and *Vmax* are inversely related[2,5,7]. We have recently shown that changing the activating [Ca^{2+}] altered *Vmax* and *a*/*Po* in ways that mirrored the differences seen between fibres[1]. Given these observations, it seems likely that differences in calcium sensitivity between fibres might cause the differences in their contractile properties. In this respect oxidative modification of muscle fibres is particularly interesting, as it affects the calcium sensitivity of fibres[8-10]. Muscle fibres exposed to hydrogen peroxide (H_2O_2) have shown reductions in maximum isometric tension[8,11,12] and in maximal velocity of shortening[10], which may be due to modifications to the myosin head[10,12]. Oxidative modification of the myosin head has been suggested to cause a reduction in force per cross-bridge[11].

To our knowledge no study has reported the effects of oxidative modification on the shape of the force-velocity relationship, an important determinant of muscular power. In the Huxley (1957) model, the shape of the relationship is given by (f+g_1)/g_2,

The authors have no conflict of interest.

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Edited by: F. Rauch
Accepted 4 November 2010

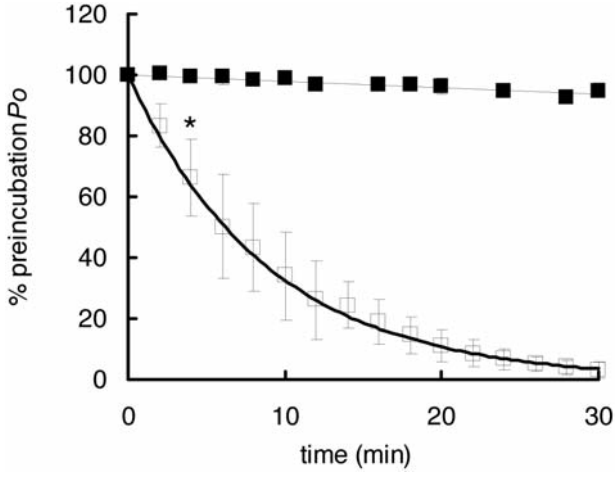

Figure 1. Effect of incubation in 50 mM H_2O_2 on maximum isometric tension. Filled squares, control fibres (n=4). Open squares, H_2O_2-treated (Mean±SD, error bars hidden when smaller than symbol size.) (n=3-5); * indicates two groups significantly different from 4 min onwards (P<0.05).

where f and g_1 are the rates of attachment and detachment, respectively, of cross-bridges in positions where they generate positive force and g_2 is the rate of detachment of cross-bridges which impede movement and produce negative force. Reducing the activating calcium led to a decrease in (f+g_1), probably due to a decrease in f, while at the same time g_2 also decreased[1]. The effect on curvature, which is given by (f+g_1)/g_2, was that there was little change for fibres with a relatively high $Vmax$ but (f+g_1)/g_2 increased for slower fibres (i.e. curvature was less), thus tending to preserve muscle power.

Given the links between oxidative modification and calcium sensitivity on the one hand, and between calcium, curvature and power on the other, the purpose of the study was to investigate the effects of oxidative modification on the force-velocity relationships of skinned muscle fibres. The hypothesis was that exposure to H_2O_2 would cause the fibre contractile properties, when activated in saturating calcium, to change in the same way as when a control fibre is submaximally activated with low calcium solutions. If so, it suggests that oxidative modification is one possible mechanism underlying the differences in contractile properties that we have observed between fibres of the same MHC composition[2].

Methods

Muscle samples

Muscle samples were obtained from young male Wistar or Sprague-Dawley rats used for other research projects approved by the local animal research ethics committees of Vrije University of Amsterdam or the University of Manchester. The rats were killed humanely using approved schedule 1 methods by either cervical dislocation (Sprague-Dawley rats) or by an overdose of pentobarbital (i.p. 50 mg·kg^{-1}) (Wistar rats), after which the soleus muscles were excised. Single fibres were dissected from the muscle samples and the baseline force-velocity relationship determined as described in[2,13].

Solutions

Solutions have been described previously[1,5]. All concentrations are given as mmol·L^{-1}. Relaxing solution: MgATP, 4.5; free Mg^{2+}, 1; imidazole, 10; EGTA, 2; KCl, 100; with the pH set to 7.0 using KOH. Low EGTA solution was the same as the relaxing solution except that EGTA was 0.5 mM. The pCa [-log[free Ca^{2+}]) of the activating solution was 4.5 and contained MgATP, 5.3; free Mg^{2+}, 1; imidazole, 20; EGTA, 7; creatine phosphate, 19.6; KCl, 64; pH 7.0. In addition a relaxing solution containing 50 mM H_2O_2 (H_2O_2+Relax) was also made, using a 30% (w/w) aqueous solution (Sigma-Aldrich, St Louis, MO, USA).

Contractile Measurements

Time course of oxidative modification

To establish the effects of H_2O_2 on maximum isometric tension over time, fibres were immersed in H_2O_2+Relax solution for 2 min, transferred to low EGTA for about 15 s, and then activating solution (pCa 4.5, but no H_2O_2) until maximum isometric tension was reached, before being returned to H_2O_2+Relax. The procedure was repeated for up to 30 min or until tension was negligible (Figure 1). Control fibres subjected to the same programme of repeated measurement showed between 0 and 10% loss of isometric force over the same time period.

Force-Velocity Relationships

To establish the effects of H_2O_2 on force-velocity relationships, a second protocol was followed (Figure 2). Fibres were subjected to isotonic shortening tests as described previously[5,14]. Fibres were transferred from relaxing solution to maximal activation solution (pCa 4.5). When the isometric force had reached a plateau the fibre was subjected to four sequences of four isotonic shortening steps before returning to the relaxing solution. Control fibres were subjected to four such sets, with the fibre incubated in normal relaxing fluid for 4 min between sets. Fibres treated with H_2O_2 were subjected to two sets, with incubation in relaxing solution between sets, followed by a 4-min incubation in H_2O_2+Relax, then a further two sets. A 4-min incubation period with H_2O_2 was chosen as the time course of force loss during incubation with H_2O_2 suggested isometric force would fall by about 35% after this time, a significant decline but with sufficient force remaining to run the isotonic tests successfully.

Rate of force redevelopment

Soleus fibres from a young-adult Sprague-Dawley rat were used to determine the effect of oxidative modification on the rate of force redevelopment (ktr). Fibres subjected to oxidation were initially immersed in H_2O_2+Relax for 4 min prior to com-

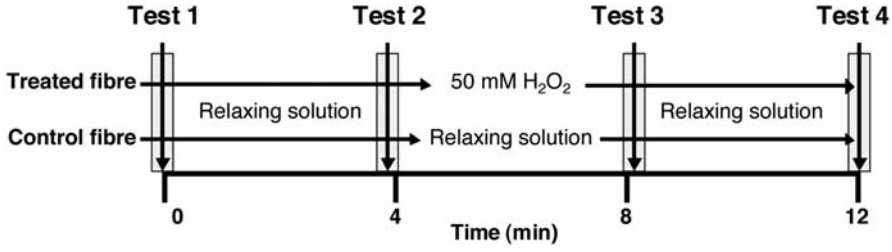

Figure 2. Testing protocol to determine the effect of H_2O_2 treatment on force-velocity characteristics. Muscle fibres were tested four times, being transferred to activating solution, as indicated by the shaded bars. The treated fibres were incubated in 50mM H_2O_2 for 4 min between Tests 2 and 3.

		Po (N·cm^{-2})	***Vmax*** (FL·s^{-1})	***(f+g$_1$)*** (s^{-1})	***g$_2$*** (s^{-1})	***(f+g$_1$)/g$_2$***	**Peak Power** (W·kg^{-1})
Pre-incubation	Mean	9.56	0.50	2.91	14.98	0.197	3.80
	SD	2.01	0.09	0.40	2.76	0.019	1.22
Post incubation	Mean	7.78	0.40	2.27	11.88	0.195	2.26
	SD	1.87	0.08	0.35	2.42	0.026	0.75
% change	Mean	-19*	-21*	-22*	-21*	-1	-39*
	SD	4	6	4	6	10	12
** P≤0.001. Pre-incubation values are averaged for the 1st and 2nd set of isotonic tests; post-incubation values are averaged for the 3rd and 4th set of isotonic tests.*							

Table 1. Effects of 4 min incubation in 50 mm H_2O_2 on the Huxley rate constants and *Vmax* of type 1 fibres (*n*=10).

mencing measurements, i.e. no measurements were taken pre-treatment with H_2O_2. Each fibre was maximally activated and subjected to one series of isotonic shortening tests, then returned to normal relaxing solution. To measure the *ktr* the fibre was transferred again to maximal activating solution and allowed to develop peak force before being rapidly released by 20% fibre length and, after 15 ms, being rapidly restretched to the original length as described in[1,13].

Myosin heavy chain composition of single fibres

After contractile measurements had been completed the single fibres were dissolved in Laemmli sample buffer, boiled for 2 min and myosin heavy chains (MHC) separated by SDS-PAGE as described previously[5].

Data analysis

Data for force and length were analysed by fitting least squares linear regressions to the last 100 ms of the length trace as a function of time, for each step, yielding 16 data velocity points for each fibre. The force and velocity data from the isotonic shortening tests was fitted to the Huxley model (1957) using the equation:

$$P/Po = 1-(1-exp(-\Phi/V))\cdot V/\Phi\cdot(1+((f+g_1)/g_2)^2\cdot V/2\Phi)$$

where f, g_1 and g_2 are rate constants (see Introduction). $\Phi = (f+g_1)\cdot h$, where h is a constant related to the distance over which a cross-bridge may act. A value of 0.0335 for h was chosen to give a mean value for $(f+g_1)$ of 2.9 s^{-1} for fibres prior to treatment with H_2O_2. This value of 2.9 s^{-1} was the mean *ktr* value for control fibres, where *ktr* is thought to represent the sum of the rate constants for attachment and detachment, similar to the Huxley value of $(f+g_1)$[15]. Data were fitted to the equation using a Matlab (v7.1, The Mathworks Inc., Natick, MA, USA) programme, giving values for $(f+g_1)$, g_2 and *Vmax*.

For each *ktr* test, data points for the recovery of force were fitted to a single exponential using non-linear least squares regression (Solver, Microsoft Excel) giving the apparent rate constant *ktr*.

Data were rejected if the isometric force decreased by more than 10% over the course of the four sequences of isotonic shortening contractions or by more than 10% for the *ktr* measurement, the sarcomere length had changed by more than 0.1 μm, or if the R^2 values were less than 0.96 when fitting the data to the Huxley equation or, in the case of *ktr* measurements, to the single exponential.

Statistical analysis

Data are expressed as mean ± S.D. Data for both control and H_2O_2-treated fibres were checked for normality using the Shapiro-Wilk test (SPSS v16.0.1) and were normally distrib-

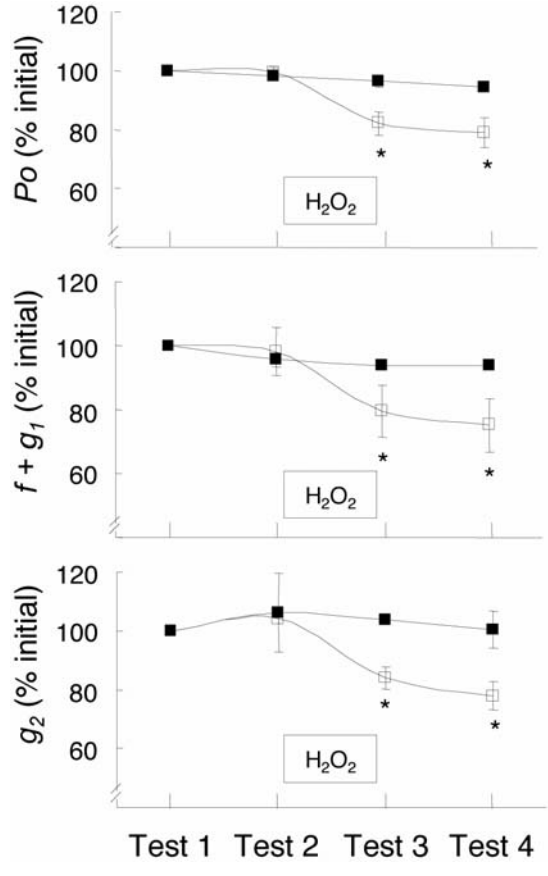

Figure 3. Changes in Po, (f+g_1) and g_2 in type 1 rat fibres as a result of 4 min exposure to H_2O_2. Each test comprised a set of isotonic shortening tests. Treated fibres were subjected to 4 min of incubation in H_2O_2 between Test 2 and Test 3. Black filled squares, control fibres (n=3); open squares, H_2O_2-treated fibres (n=10). Mean±SD, error bars hidden when smaller than symbol size. * declined compared to previous series (P<0.001).

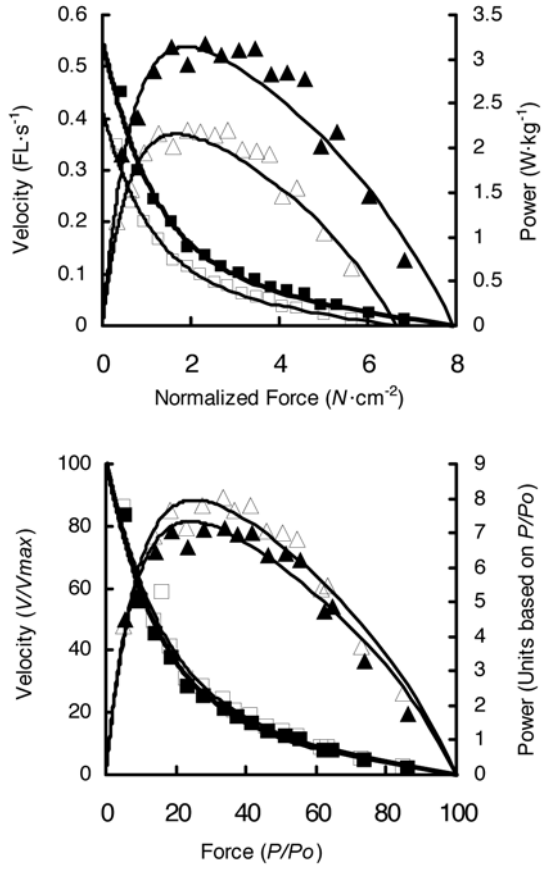

Figure 4. The effects of 4 min incubation in H_2O_2 on the force-velocity relationship in a typical fibre. **A.** Force-velocity and force-power curves before and after incubation. **B.** Force-velocity data normalized to maximum isometric force (Po) and maximal shortening velocity ($Vmax$) to show the effect on the curvature of the relationship. Filled symbols, pre incubation; open symbols after incubation. Squares indicate velocity for a given force; triangles, power for a given force.

uted. A mixed design analysis of variance (ANOVA) was used to analyse differences between groups (H_2O_2 treated or control) over time (four test periods). Simple effects were analysed using Student's t-tests (either paired or unpaired as appropriate). Differences were taken to be significant when $P \leq 0.05$.

Results

Oxidative modification and the force-velocity relationship

Six control and 15 H_2O_2-treated fibres were subjected to four sets of isotonic shortening tests. Subsequently, three control and 10 H_2O_2-treated were identified as MHC type 1 fibres. The other fibres were either type 2a or hybrid type 1-2a and the results for those fibres are not reported here.

Prior to incubation in H_2O_2, the H_2O_2-treated group had higher baseline values of (f+g_1) than the control group (Test 1, P=0.035; Test 2, P=0.020). $Vmax$ and Po were similar, although Po tended to be higher in the H_2O_2-treated group too (Test 1, P=0.077; Test 2, P=0.063). Consequently, changes of Po and the calculated values of the Huxley rate constants normalized to Test 1 are shown in Figure 3. For the fibres exposed to H_2O_2 the Huxley average rate constants for the preincubation Tests 1 and 2, and Tests 3 and 4 are given in Table 1 together with the change in peak power. In both control and H_2O_2-treated fibres there were no significant changes in these parameters between Tests 1 and 2. In control fibres, there was no change between Tests 2 and 3. In contrast the fibres treated with H_2O_2 for 4 min immediately after Test 2, showed falls of around 20% in all three measures [Po, (f+g_1), and g_2] at Test 3 (P<0.001) with a further small decline by Test 4 (P<0.05).

Figure 4 illustrates the decrease in Po, $Vmax$ and peak power but with little change in curvature of the force-velocity relationship for a typical fibre.

Individual values for (f+g_1) and g_2 are plotted in Figure 5A where it can be seen that there is a linear relationship between the two with an intercept on the ordinate giving a hypothetical value for (f+g_1) of 0.68 when g_2=0. Although incubation with H_2O_2 caused a reduction in both (f+g_1) and g_2, the relationship

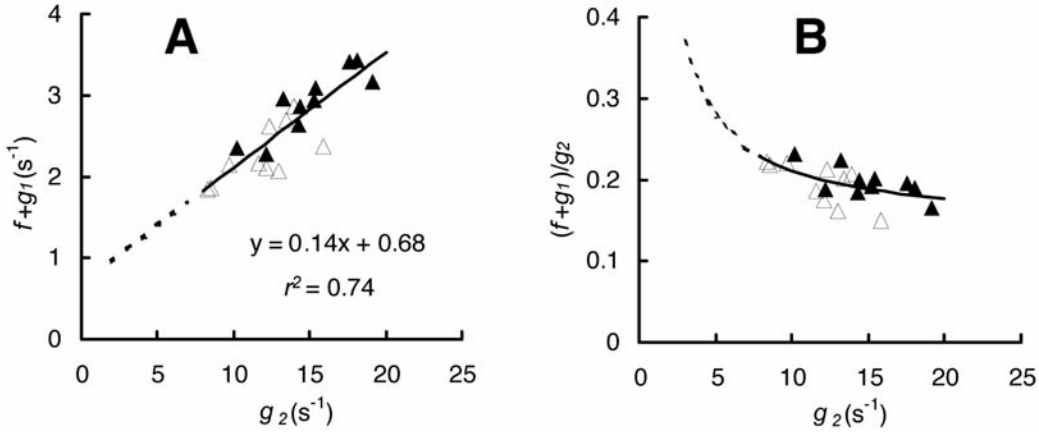

Figure 5. Changes to rate constants following a four minute incubation in 50 mM H_2O_2. **A.** The relationship between g_2 and (f+g_1). **B.** The relationship between curvature (i.e. (f+g_1)/g_2) and g_2. Filled symbols, values prior to treatment with H_2O_2 (average of 1st and 2nd tests); open symbols, values post-incubation in H_2O_2. The regression line in A is from both pre-and post-incubation data points. The line in B is that derived from the regression line in A.

		Po (N·cm^{-2})	***Vmax*** (FL·s^{-1})	(f+g_1) (s^{-1})	g_2 (s^{-1})	(f+g_1)/g_2	***ktr*** (s^{-1})	***Peak Power*** (W·kg^{-1})
Control	Mean	12.5	0.52	2.52	15.5	0.162	2.90	4.12
(n=4)	SD	4.0	0.06	0.33	1.7	0.014	0.12	1.48
H_2O_2 treated	Mean	12.7	0.46	1.95	13.6	0.144	2.20	3.26
(n=5)	SD	2.1	0.07	0.25	2.0	0.016	0.11	0.76
% difference	Mean	+1	-12	-23*	-12	-11	-24**	-21
* P=0.021; ** P<0.001								

Table 2. Values of Huxley rate constants and *ktr* values of fibres incubated for 4 min in 50 mm H_2O_2 compared to untreated control fibres.

remained the same with the data points moving to the left along a regression line fitted to all data points. The consequence of the relationship shown in Figure 5A for curvature of the force-velocity relationship is shown in Figure 5B where the ratio (f+g_1)/g_2, reflecting curvature in the Huxley model, is plotted against g_2, with higher values of (f+g_1)/g_2 indicating less curvature.

The rate of force redevelopment (ktr)

Rate constants for force redevelopment were obtained for four control and five H_2O_2-treated fibres. The *ktr* values for oxidised fibres were significantly slower than those for untreated fibres (2.20±0.11 s^{-1} compared to 2.90±0.12 s^{-1}, P<0.001). The Huxley rate constants calculated from the isotonic tests preceding the *ktr* measurement are shown in Table 2. Values of (f+g_1) were lower in H_2O_2-treated fibres compared to untreated fibres (P=0.021). The rate constant g_2 was comparable between groups although there was a tendency for it to be lower in the H_2O_2-treated group (P=0.170). *Po* was similar in treated and untreated fibres.

Discussion

The results presented here show that 4 min incubation in 50 mM H_2O_2 caused decreases of approximately 39, 19 and 21% in peak power, *Po* and *Vmax* respectively, with virtually no change in (f+g_1)/g_2, an indicator of the curvature of the force-velocity relationship.

Four min incubation with 50 mM H_2O_2 was chosen, as it resulted in significant changes in contractile properties whilst still maintaining sufficient function to make meaningful measurements feasible (Figure 3). Other studies using similarly high concentrations (5-50 mm) also showed marked reductions in *Po* and maximum shortening velocity[9-11], but to our knowledge this is the first study which has reported the effect of oxidation on curvature.

The reductions in *Vmax* and, possibly, curvature reflect changes to the rate constants for attachment and detachment of cross-bridges. In terms of the Huxley rate constants, oxidation caused a reduction of around 20% in (f+g_1) and g_2 (Figure 3 and Table 1). On the assumption that *ktr* represents the apparent

turnover rate of cycling cross-bridges (f_{app}+g_{app})[15] and that this is equivalent to (f+g_1), the *ktr* experiments confirmed the reduction in (f+g_1), where *ktr* values of oxidised fibres were on average 24% slower than those of untreated fibres (Table 2).

These changes in *Po*, *Vmax* and *ktr* are similar to those that occur when the activation level is reduced[1]. It is possible, therefore, that the changes to the contractile properties arising from oxidation are caused by some process effectively leading to reduced activation. A change in calcium sensitivity might change the extent of activation and several research groups have shown that calcium sensitivity of skinned muscle fibres is changed by exposure to H_2O_2. Reduced sensitivity, measured by a reduction in pCa_{50}, (the negative logarithm of the free calcium concentration at which 50% of maximum isometric force is produced) has been observed with 20-30 min exposure to high concentrations (10-50 mm) of H_2O_2, in conjunction with a reduction in *Po*[9,10].

The effects of exposure to H_2O_2 on (f+g_1) and g_2 (Figure 5A) are similar to those we found with reduced activation[1] although not as large. Both declined linearly and the slope of the relationship is similar and the intercept on the ordinate is of the same order in the two studies. However, whereas this decline resulted in a decrease in curvature when activating calcium was reduced, there was no significant change as a result of oxidation (Table 1). This difference can be explained by a difference in the magnitude of the effects of oxidation versus submaximal activation on (f+g_1) and g_2. The linear relationship between (f+g_1) and g_2 has a positive intercept (Figure 5A), so that at high values of g_2 the ratio (f+g_1)/g_2 is relatively constant, but for low values the ratio rises rapidly as g_2 declines. Activation in pCa 5.6 caused a relatively large change in g_2, hence leading to an increase in the ratio (f+g_1)/g_2 (decrease in curvature). In contrast, oxidation has a smaller effect on g_2 so that the fibres remain on the relatively linear portion of the range between curvature and g_2 (Figure 5B).

In our previous work[1] we discussed how the rate constants f and g_2 might be inter-related, with both declining at reduced activation. The suggestion was made that this might be due to a slowing of cross-bridge transition from low to high force states leading to an accumulation of low-force cross-bridges. This, in turn, would lead to a decrease in the apparent rate constant f and the internal load would reduce shortening velocity, an idea first proposed by Moss[16], and be apparent as a decrease in g_2. However, given that reduced [Ca^{2+}] appears to reduce stiffness, an indicator of the number of attached cross-bridges, in proportion to force[17] a shift in the proportion of cross-bridges in low force states seems unlikely.

A single bout of exposure to H_2O_2 appears to have resulted in a process of oxidative modification, with a further slight decline in (f+g_1) and g_2 at test 4 compared to test 3 (Figure 3). Why this later decline occurred is unclear.

Clearly 50 mM H_2O_2 is a great deal higher than physiological levels in working muscles which are reported to be 10^{-9}-10^{-7}M[18]. However, Lamb and Posterino (2003)[9] argue that it is still possible to infer *in vivo* consequences from experiments using significantly higher concentrations, since the presence of iron in the body greatly enhances the potency of H_2O_2. A useful follow-up to this study would be to look at the effects of oxidation on the power of isolated muscle fibres using a much lower concentration of H_2O_2 together with myoglobin. This approach has been used by Murphy et al[12] who observed a reduction in calcium sensitivity in both soleus and *extensor digitorum longus* (EDL) fibres and a reduction in *Po* in EDL fibres with a 20-min incubation in 300 μM H_2O_2 plus 0.5 mM myoglobin.

It would be particularly interesting to see whether the effects of oxidation on curvature are reversible. Other research groups have looked into whether or not exposure to a reducing agent can reverse the effects of oxidation on force and on velocity. The effects have been partially or fully reversed in some cases, depending mainly on the severity of exposure to H_2O_2 (concentration and duration). For example, the reductions in *Po* and maximum unloaded shortening velocity of rabbit psoas fibres caused by 30 min exposure to 5mM H_2O_2 were completely reversed by 15 min incubation in 100 mM dithiothreitol. However, following 30 min exposure to 50 mM H_2O_2, the same reducing treatment had no effect on velocity and only partially reversed the reduction in *Po*[10]. Other factors such as fibre type and whether or not a fibre is activated prior to incubation in the reducing agent have also been shown to affect reversibility[12]. An insight into the reversibility of any effects on curvature would be useful because, as demonstrated here, this parameter provides an insight into the balance between the rate constants, something that *Po* and *Vmax* on their own alone cannot provide.

In summary, this study found that exposure of type 1 fibres to H_2O_2 caused marked reductions in peak power, *Po* and *Vmax*. The decline seen in the Huxley rate constants (f+g_1) and g_2 mirror the decline seen in these rate constants when fibres are activated submaximally. It is suggested that this implies that oxidative modification affects the contractile properties of a fibre by reducing their effective level of activation. It also leads to the possibility that oxidation may be a cause of the wide variation in contractile properties observed in fibres of the same MHC type so that differences in the pattern of use or location within the muscle may result in different extents of cumulative oxidative modification.

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