APPS/PSNZ Meeting - Sydney 2003

Free Communications 5: Skeletal muscle

Tuesday 30th September 2003

Chair: Graham Lamb

Variations in myosin expression along the length of orbital fibres in the rabbit extraocular muscle

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Extraocular muscles (EOM) have two layers of muscle fibres with different functions: orbital fibres that control the position of the recently discovered soft tissue pulleys (Demer *et al*., 2000), and global fibres that rotate the globe. Pulleys make the axes of action of EOMs depend on eye orientation, and this is thought to provide a simple mechanism for implementing Listing's law governing eye rotations. Both layers have SIFs (singly innervated fibres) and MIFs (multiply innervated fibres), with ultrastructural features resembling amphibian fast twitch and slow-tonic fibres, respectively. EOM fibres express 9 myosin heavy chain (MyHC) isoforms, comprising those in developing and adult limb and cardiac muscles, and 2 EOM-specific isoforms, EO-MyHC and slow-tonic MyHC. Orbital fibres display a systematic variation of MyHCs along their length, correlated with ultrastructural features, but earlier studies were unable to specify the precise MyHC isoforms involved. We use here a battery of monoclonal antibodies capable of unambiguously identifying each of the 9 MyHCs to study MyHC changes in serial sections of rabbit superior rectus muscle by immunohistochemistry.

According to ultrastructural criteria (Davidowitz *et al*., 1977), there are three major orbital fibre types: the oSIF, the coMIFs (orbital MIF of constant diameter); and the voMIFs, which vary in diameter from 5μm along the middle portion of their length to around 15 μm in their ends. The oSIFs and coMIFs are short, whilst the voMIFs are the longest. Orbital MIFs have an 'en plaque' neuromuscular junction in addition to distributed 'en grappe' endplates in global MIFs.

We show that variations in MyHC expression in orbital fibres closely parallel structural variation along the length. These changes occur at three sites: (1) At the EPZ, the oSIFs express EO-MyHC, the fastest MyHC, associated with high sarcoplasmic reticulum (SR) and mitochondrial volume. On either side of the EPZ, these fibres express the slower 2A and/or embryonic MyHCs, with decreased SR and mitochondrial volume. (2) The coMIFs and voMIFs at the EPZ express α-cardiac MyHC, the fastest of the slow MyHCs, where the ultrastructure is fast twitch. They continue to show twitch ultrastructure on either side of the EPZ, where they coexpress α-cardiac and embryonic MyHC. (3) In the distal quarter of the orbital layer, the oSIFs and coMIFs end, presumably by inserting onto the pulley, the orbital layer is entirely made up of voMIFs. Here the fibres mainly co-express slow-tonic and embryonic MyHC and show ultrastructural features of amphibian slow-tonic fibres. In the far proximal end of the muscle, oMIF mainly express embryonic MyHC with a small proportion of fibres co-expressing slow-tonic MyHC.

We propose that only the oSIFs and coMIFs insert into the pulleys and actively translocate them during saccades. Forward translocation of pulleys is achieved by passive stretching due to contraction of the antagonist, the presence of the very fast EPZ region permitting sudden collapse of tension necessary for rapid repositioning of the pulleys. The voMIFs insert onto the globe. The slow-tonic MyHC may provide ripple-free tension to hold the eyeball steady during a gaze, and the faster narrow segment may be a specialisation to allow for rapid relaxation and fibre lengthening during a change of gaze involving contraction of an antagonist.

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Fibre types in rat laryngeal muscles and their transformations following denervation and reinnervation

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Intrinsic laryngeal muscles cricothyroid (CT) and thyroarythenoid (TA) differ in myosin expression. CT expresses limb myosin heavy chains (MyHCs) while TA expresses a MyHC found in extraocular (EO) muscles (Lucas *et al.*, 1995), in addition to limb isoforms. A definitive classification system for laryngeal muscle fibre types does not exist at present. Earlier studies on the effects of denervation (Shiotani & Flint, 1998) and reinnervation (Shiotani *et al.*, 2001) on the MyHC profiles of whole laryngeal muscles are suggestive of neural influence on MyHC expression, but fibre type transformation at the cellular level has not been shown.

Immunohistochemical analyses with highly specific monoclonal antibodies (mAbs) against various MyHCs were used to study muscle fibre types in rat CT and TA, and to investigate whether nerves to laryngeal muscles control MyHC expression. CT was found to have the full complement of limb fibre types. TA had three major fibre types based on MyHC composition: 2b/eo, coexpressing 2B and EO MyHCs, 2x/2b, coexpressing 2X and 2B MyHCs, and 2x, expressing 2X MyHC. Type 2a and slow fibres were absent. TA consisted of two divisions: the external division (TA-X), which is homogenously 2b/eo, and the vocalis division (TA-V), composed principally of 2x and 2b/eo fibres, with a minority of 2x/2b fibres. The use of these mAbs has established the feasibility of classifying laryngeal muscle fibre types by their MyHC composition in spite of the extensive occurrence of hybrid fibres containing multiple isoforms.

The recurrent laryngeal nerve (RLN) which innervates both divisions of the TA as well as other laryngeal muscles except the CT were cut and allowed to reinnervate these muscles in 16 rats. The left RLN transection was performed on sixteen 10-week old Sprague Dawley rats. The animals were anaesthetised by intraperitoneal injection of ketamine hydrochloride (35mg/kg) and xylazine hydrochloride (5mg/kg). The TA from 4 animals were examined immunohistochemically at 2, 4, 6 and 12 weeks postoperatively. Commencing four weeks after cutting and re-uniting the RLN, numerous 2b/eo fibres in TA-X began to express 2X MyHC, while EO and 2B MyHC expression in these fibres progressively declined. By 12 weeks, 16.5± 2.5(SE)% of fibres in the TA-X were of type 2x. These findings suggest that nerve fibres originally innervating 2x fibres in TA-V and other muscles had randomly cross-innervated 2b/eo fibres in the TA-X and converted them into 2x fibres. We conclude that MyHCs in laryngeal muscle fibres are subject to neural regulation, in common with limb and jaw muscles.

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There is no difference in the net efficiency of fast- and slow-twitch mouse muscles

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It is commonly accepted that slow-twitch muscles are more efficient than fast-twitch muscles; that is, slow twitch muscles convert a greater fraction of the energy they use into mechanical work. Evidence supporting this idea comes from two types of experiment. First, humans with a greater fraction of slow-twitch fibres are more efficient when cycling on an ergometer (Coyle *et al.*, 1992) and, second, isolated preparations of slow-twitch muscle use less high energy phosphate per unit work performed than fast-twitch preparations (Barclay, 1996). The human experiments have the drawback that it is difficult to make inferences about muscle efficiency from measurements of whole body O_2 consumption. The isolated muscle experiments are difficult to relate to *in vivo* efficiency because: (1) efficiency was measured only during shortening, rather than over complete cycles of shortening and lengthening; and (2) because the indices of energy cost used did not encompass oxidative recovery processes. In the only study comparing efficiency of fast and slow muscles that used cyclic contractions and in which O_2 consumption was used as the index of energy use, slow-twitch rat muscles were found to be less efficient than fast-twitch muscles (Heglund & Cavagna, 1987). However, that study used a temperature of 20°C rather than physiological temperature.

The aim of this study was to measure efficiency of isolated fast- and slow-twitch muscles using a pattern of activity similar to that occurring *in vivo*, using the energetic equivalent of O_2 consumption as the index of energy cost and performing the experiments at a temperature of 35°C.

Experiments were performed *in vitro* using bundles of muscle fibres from the slow-twitch soleus and fast-twitch EDL muscles of mice. Muscles were dissected from mice that had been killed by inhalation of CO_2. Efficiency was calculated from measurements of work output and total heat production during and after a series of 20 contractions. The contraction protocol consisted of a realistic, cyclic pattern of muscle length changes with a brief contraction in each length cycle. Twenty contractions were performed at a frequency of 3.4 Hz. Net mechanical efficiency was defined as the ratio of work output to the total, suprabasal enthalpy output and enthalpy output was the sum of the heat and work output.

There was no difference in the maximal net efficiency of fast- and slow-twitch mouse muscles. Maximum efficiency of soleus muscles was 13.9 ± 0.8 % ($n = 6$) and of EDL muscles was 13.5 ± 0.5 % ($n = 6$).

This result suggests that any relationship between human efficiency and fraction of slow-twitch fibres is not a reflection of an intrinsic difference in efficiency of fast and slow muscle fibres.

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Treatment with the β_2-agonists formoterol or salmeterol produce greater muscle hypertrophy in rats than fenoterol

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Although traditionally administered at low doses for treating asthma, at higher doses, β_2-adrenoceptor agonists (β_2-agonists) have potent muscle anabolic effects. As such, β_2-agonists may have therapeutic potential for pathologies where muscle wasting is indicated, such as cancer cachexia, muscular dystrophy and age-related muscle wasting (sarcopenia). Before these drugs can be considered as legitimate therapies, some safety concerns, especially their effects on the heart, need to be considered. The β_2-agonists, formoterol and salmeterol were originally developed to increase the duration of bronchodilation. Previous studies have shown formoterol and salmeterol to have a duration of action of four and eight times greater, respectively, than the most widely used asthma drugs (Anderson, 1993). We have previously shown that the (short-acting) β_2-agonist fenoterol has greater anabolic effects on skeletal muscle than the most widely described, in relation to skeletal muscle, β_2-agonist, clenbuterol (Ryall *et al.*, 2002). In the present study, we tested the hypothesis that due to their long duration of action, chronic administration of salmeterol and formoterol would produce greater skeletal muscle hypertrophy than fenoterol. One of our research goals is to optimise the safe and effective use of β_2-agonists to ameliorate muscle wasting in a number of pathologies.

Fenoterol, formoterol and salmeterol (kindly supplied by Astra-Zeneca) were administered to male Fischer 344 rats (12 weeks/age, body mass, 265g) at one of five different doses (0.025 - 2 mg/kg/day) for four weeks. Fenoterol and formoterol were administered by daily i.p. injection in saline, and compared to a control group receiving an equivolume of saline. Due to its highly lipophillic nature, salmeterol was administered via a daily i.p. injection in a lipid vehicle, and compared to a control group receiving an equivolume of lipid vehicle. The rats were deeply anaesthetised (sodium brietal, 60 mg/kg), and the heart, and the EDL and soleus hindlimb muscles were surgically excised, weighed, and then stored for histological analyses.

The rank order of efficacy (E_{max}), based on skeletal muscle hypertrophy (β_2-agonist induced increase in mass above control), was salmeterol = formoterol >> fenoterol. Salmeterol had an E_{max} at a dose of 1 mg/kg/day, increasing EDL, soleus and heart mass, 39, 28 and 25% above values for lipid vehicle control. Formoterol had an E_{max} at a dose of 0.5 mg/kg/day, increasing EDL, soleus and heart mass, 36, 26 and 26% above values for saline control. Fenoterol had an E_{max} at a dose of 2 mg/kg/day, increasing EDL, soleus and heart mass, 25, 14 and 23% above values for saline control. At the lowest dose examined (0.025 mg/kg/day) formoterol exhibited the greatest hypertrophy of both skeletal and cardiac muscle compared to values for saline control, (19, 13 and 12% greater for EDL, soleus and heart, respectively).

Our findings indicate that the β_2-agonists, formoterol and salmeterol, have anabolic effects on muscle and produce greater muscle hypertrophy than fenoterol. Further research is needed to examine the effect of these drugs on skeletal and cardiac muscle function before their full therapeutic potential can be realised.

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Supported by the Muscular Dystrophy Association (USA), the Rebecca L. Cooper Medical Research Foundation, and The University of Melbourne.

JGR is supported by a Postgraduate Scholarship from the National Heart Foundation of Australia

Ca^{2+} handling properties of mechanically skinned fibres from fast and slow muscles of adult and old rats following chronic fenoterol treatment

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Aging is associated with a progressive loss of motor function, a slowing of muscle movements, and a decline in muscle strength. These age-related changes in skeletal muscle contribute to the increased incidence of fall-related injuries in the elderly, resulting in a loss of functional independence. β_2-agonists (such as fenoterol) have potent muscle anabolic effects and we have recently demonstrated that four weeks treatment with fenoterol is sufficient to ameliorate the age-related muscle weakness and slowing of contraction in rats (Ryall *et al.*, 2002). In another study we demonstrated that aging deleteriously affects aspects of excitation-contraction coupling and sarcoplasmic reticulum (SR) function in mechanically skinned fast muscle fibres from aged compared with adult mice (Plant & Lynch 2002). It is not known whether fenoterol treatment would affect these properties in mechanically skinned fast and slow muscle fibres from aged rats.

We tested the hypothesis that four weeks fenoterol treatment would alter SR Ca^{2+} handling properties of mechanically skinned skeletal muscle fibres differently in adult and old F344 rats. Adult (16 months/age) and old (28 months/age) rats were treated daily with either fenoterol (1.4 $mg.kg^{-1}day^{-1}$, i.p.) or saline vehicle, for four weeks. Following treatment, rats were anaesthetised with sodium pentobarbitone (60 $mg.kg^{-1}$, i.p.) and the fast-twitch extensor digitorum longus (EDL) and predominantly slow-twitch soleus muscles excised carefully to prepare mechanically skinned fibres. Fibres were tested according to the methods we have described in detail previously (Plant & Lynch, 2002).

Preliminary findings indicate no age-related changes in normalised SR Ca^{2+} reloading or leak of Ca^{2+} from the SR. Fenoterol increased leak of Ca^{2+} from the SR in EDL but not soleus muscle fibres from adult and old rats. Rate of Ca^{2+} reloading was decreased with fenoterol treatment in EDL muscle fibres from both adult and old rats, but soleus muscle fibres from adult and old rats were not affected. These findings suggest that fenoterol's effects are similar in mechanically skinned fibres from adult and old rats. The effects of fenoterol on depolarisation-induced force responses in mechanically skinned fibres has yet to be examined.

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Supported by grants from the Muscular Dystrophy Association (USA)