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Expression of myosin isoforms in masseter of human subjects with malocclusions

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Samples are obtained from masseter of subjects undergoing orthognathic surgery for treatment of malocclusion, snap frozen for myosin immunostaining to identify fibre types and processed in parallel with samples of limb muscle taken from the margin of excised soft tissue tumours. Sample collection from consenting subjects was approved by the local Research Ethics Committees of Guy's Hospital (limb samples) and Lille (masseter). Cross-sectional areas of typed fibres (identified according to Sciote et al. 1994) are measured to work out their relative 'occupancy' (% area) of the biopsy and serial sections are collected for quantification of myosin heavy chain gene expression by RT-PCR using isoform-specific probes. Values of myosin message in each reaction are calculated as pg of amplified DNA per ng of internal 18S RNA, and the relative content of each individual MHC isoform RNA is obtained as a percent of the total of all myosin message in that sample. Malocclusion is diagnosed from sagittal (class I, II and III) and vertical dimensions (Open, Normal and Deep) from lateral cephalograms.

The relative abundance of the mRNAs for each isoform was compared with an estimate of the corresponding myosin protein content obtained from the fiber type occupancy values, in the form of a simple ratio (protein:message). The Table shows mean ratios for masseter samples separately for the 9 different craniofacial groups and for the limb muscle samples.

Values are close to 1 for limb muscle samples, but show more mismatch in masseter samples where there is generally a 'deficit' of type I message (ratios greater than 1) and a very variable 'excess' of message for neonatal and atrial isoforms in most cases. We conclude that in subjects with malocclusion, translational control of myosin expression is different in masseter muscle compared with limb muscles.

Protein: mRNA (p:m) ratios (±s.e.m.) for myosin heavy chain isoform

Malocclusion	n	type I ratio p:m	type IIA + IIX ratio p:m	neonatal ratio p:m	atrial ratio p:m
I-open	1	1.6	0.5	2.46	0.49
I-normal	3	1.69±0.55	0.76±0.16	0.01±0.01	0.56 ±0.33
I-deep	1	7.45	0.41	0.78	0.09
II-open	8	1.12±0.08	2.17±0.51	0.18±0.07	0.63±0.26
II-normal	4	1.44±0.29	0.90±0.31	0.06±0.04	1.0±0.63
II-deep	5	1.43 ±0.28	1.16±0.35	0.37 ±0.2	0.51±0.22
III-open	4	1.14±0.11	0.92±0.17	0.34±0.19	0.67±0.48
III-normal	4	1.49 ±0.11	1.12 ±0.22	0.05±0.05	0.52 ±0.2
III-deep	1	1.45	1.28	#	0.54
limb	6	0.91±0.13	1.32±0.18	~	~
		#Message present but no protein; ~neither message nor protein.			

Sciote et al. (1994). J. Neurol. Sci. 126, 15-24.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

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The use of *in vivo* microdialysis to detect reactive oxygen species (ROS) in skeletal muscle extracellular fluid

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To allow differentiation between the pathological and physiological role that ROS play in skeletal muscle, it is essential to identify and quantify these species. We have previously demonstrated increased superoxide ($O_2^{\cdot-}$) and hydroxyl (·OH) production in muscle extracellular space during exercise (McArdle *et al.*, 2001;2004). These methods involved the assumption that $O_2^{\cdot-}$ reduces cytochrome c (CC) and that ·OH causes the hydroxylation of salicylate; however, nitric oxide (NO) and peroxynitrite ($ONOO^-$) may also contribute to these processes (Murrant and Reid, 2001). This study examined the effects of specific ROS inhibitors on the reduction of CC and hydroxylation of salicylate in vivo using microdialysis.

24 male C57Bl/6 mice were allocated into 4 groups. Group 1 received an IV injection of 50mg/kg b.wt of the NO synthase inhibitor NG-nitro-L-arginine methyl ester (L-NAME). Group 2 received 10mg/kg of the iron chelator desferrioxamine mesylate (desferal) which prevents the formation of ·OH via Fenton chemistry, and group 3 received 5,000U/kg superoxide dismutase (SOD), which catalyses the dismutation of $O_2^{\cdot-}$, both by IP injection. Group 4 were untreated controls. 30-min post drug administration, mice were anaesthetised with sodium pentobarbitone (5-10mg/100g IP) and supplemental doses were given as required to maintain a sufficient depth of anaesthesia. Whilst anaesthetised, 2 microdialysis probes were placed into the *gastrocnemius* muscle and perfused with salicylate or CC in saline. $O_2^{\cdot-}$ activity was analysed in the perfusates by measurement of CC reduction and ·OH activity by measurement of 2,3-dihydroxybenzoate (2,3-DHB) formed from salicylate (McArdle *et al.*, 2004). Following 1 hr of baseline collections, the hind-limb was subjected to a 15-min period of isometric contractions (McArdle *et al.*, 2001). Mice were humanely killed 30 min post-exercise. Data was analysed using a mixed design ANOVA ($P<0.05$).

The reduction in CC was greater in mice treated with L-NAME compared with control mice (e.g. mean resting value = 0.34 (SD 0.03) versus 0.25 (SD 0.09) nmol/15 min, $P<0.05$). The reduction of CC was attenuated in mice treated with SOD compared with control mice, (e.g. mean resting value = 0.16 (SD 0.02) versus 0.25 (SD 0.09) nmol/15 min, $P<0.05$). The hydroxylation of salicylate was attenuated in mice treated with desferal compared with control mice (e.g. mean resting value = 3.77 (SD 2.81) versus 8.28 (SD 2.50) pmol/15 min, $P<0.05$). There was no effect of L-NAME treatment on the hydroxylation of salicylate (e.g. mean resting value = 7.78 (SD 2.06) versus 8.28 (SD 2.50) pmol/15 min, $P>0.05$). Data suggest that $O_2^{\cdot-}$ is the major contributor to the reduction of CC and ·OH causes the hydroxylation of salicylate, supporting the use of these assays to detect extracellular ROS generation *in vivo* using microdialyis.

McArdle A, *et al.* Am J Physiol Cell Physiol 2001;280:C621-7

McArdle A, *et al.* Am J Physiol Cell Physiol 2004;286:C1152-8

Murrant CL and Reid MB. Microsc Res Tech 2001;55:236-48

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

C95

Delocalisation of neuronal nitric oxide synthase may result in the aberrant generation of reactive oxygen species in dystrophic mice

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Duchenne Muscular Dystrophy (DMD) is a degenerative muscle disorder that is associated with myofibre necrosis. Increased generation of reactive oxygen species (ROS) (Haycock et al., 1996) and the delocalisation of neuronal nitric oxide synthase (nNOS) from the sarcolemma (Brenman et al., 1995) are two pathophysiological events that have been reported in dystrophic muscle. The aim of this study was to investigate whether the delocalisation of nNOS in mdx mice results in aberrant ROS generation in skeletal muscle.

2-4 month old wild type (WT)(n=6) and mdx(n=5) mice were anaesthetised using sodium pentobarbital(10mg/100g) via an intraperitoneal injection. Two microdialysis probes were inserted into the gastrocnemius muscle and perfused with saline for the measurement of nitrate/nitrite formation as a measure of NO release, or cytochrome c for the measurement of superoxide (McArdle et al., 2001). Following insertion of the microdialysis probes, baseline microdialysis perfusates were collected for 1 hour to allow the baseline to stabilise. Mice were then subjected to a 15-min period of isometric contractions (McArdle et al. 2001). Perfusates were collected during contractions and over the subsequent 1-hour recovery period. Immediately following the final collection, mice were killed by cervical dislocation and muscle samples were removed for analysis of nNOS expression. Total nNOS expression in muscle homogenates was determined by western blotting, and the localisation of nNOS was assessed through immunohistochemical studies. Data was analysed using a mixed design ANOVA ($P<0.05$).

Superoxide generation during contraction was significantly increased in muscles of mdx mice compared with generation during contraction in muscles of WT mice (mean = 0.38 (SD 0.08) versus 0.25 (SD 0.11) nmol/15min, $P<0.05$). There was a trend for attenuated NO release from skeletal muscle fibres in mdx mice, although this was not statistically significant (during contraction mean = 339 (SD 154) versus 1077 (SD 339) pmol/15min, $P>0.05$). Total nNOS levels were reduced in mdx skeletal muscle homogenates. Immunohistochemical studies revealed the absence of nNOS from the sarcolemma in mdx mice. The data is consistent with the theory that the loss of nNOS into the cytosol of the skeletal muscle fibre results in an alteration of nNOS function, leading to increased superoxide release. The effects of this altered nNOS function may have potential cytotoxic consequences that may be implicated in the pathogenesis of DMD.

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Haycock J.W. et al(1996) Neuroreport 8:357-361

McArdle A. et al(2001) Am J Physiol Cell Physiol:C621-C627

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

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THE EFFECTS OF AGE ON NFAT EXPRESSION IN RAT FAST AND SLOW MUSCLES

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In a recent study, we showed that a stretch/release cycle of ~20-25% L_o leads to a marked increase in intracellular calcium levels $[Ca^{2+}]_i$ in relaxed neonatal, but not in adult, rat muscle fibre bundles; we proposed that this increase may play a role in post-natal muscle differentiation (Mutungi, et al., 2003). In cultured skeletal myocytes an increase in $[Ca^{2+}]_i$ has been found to up-regulate slow-fibre type specific gene promoters via the activation of the cyclosporin-sensitive, calcium-calmodulin regulated serine/threonine phosphatase, calcineurin and these effects are partly mediated by members of the nuclear factor of activated T-cells (NFAT) family (Chin et al., 1998). However, little is known about the expression of members of the NFAT family during post-natal differentiation. Therefore, the aim of this study was to investigate the expression of two members of this family, NFATc1 and NFATc2, in fast and slow muscles of both neonatal (1-21 days old) and adult (>30 days old) rats.

The rats were humanely killed with an overdose of sodium pentobarbitone injected intra-peritoneal. Immediately after death, the extensor digitorum longus (edl, mainly a fast muscle) and soleus (a predominantly a slow muscle) were carefully dissected. They were mounted onto cryostat chunks (half perpendicularly and the other half horizontally) and rapidly frozen in liquid nitrogen. 10-15μm thick serial sections were cut. One section from each group was stained for alkaline myosin ATPase activity (for fibre type identification) and the others for NFATc1 and c2 expression using monoclonal antibodies. Non-specific antibody binding was prevented by incubating the sections, for 30 minutes, in a blocking buffer (PBS containing 2% horse serum).

In both the fast and slow muscles, no NFATc1 expression was seen in the cytoplasm of fibres of rats <10 days old (n=4) but they all stained strongly for NFATc2. Thereafter, the expression of NFATc1 in the cytoplasm of the fibres progressively increased whereas that of NFATc2 decreased with age. Thus, in adult rats 5±0.8% of the fibres in the soleus (n=5rats) and 39.8±4.4% of the fibres in the edl (n=5 rats) expressed NFATc1 in their cytoplasm. In the same muscles 42.3±0.9% of the fibres in the soleus and 2±0.1of the edl fibres expressed NFATc1 in their nuclei. On the other hand, NFATc2 was found to be mainly expressed in the nuclei of the adult fibres with 46.4±2% of edl fibres and 57.6±0.9% of soleus showing the presence of this isoform in their nuclei. These results suggest an age and fibre type dependent expression of the two NFAT isoforms investigated. However, their physiological role in these fibres remains uncertain.

All values are mean ±S.D.

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Mutungi G *et. al.* (2003) *JPhysiol***551**, 93-102

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

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Contractile performance of isolated muscle from mice over-expressing uncoupling protein 3

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Uncoupling protein 3 (UCP3) is a mitochondrial transporter, which is specific to skeletal muscle, and allows protons to proceed through the membrane without synthesis of ATP. Transgenic mice over-expressing UCP3 (UCP3-oe) are lean despite being hyperphagic and having a normal level of locomotion (Clapham et al. 2000). Experiments on isolated muscle have shown the recovery heat production after isometric contraction is higher when extra UCP3 is present (Curtin et al. 2002), indicating inefficient production of ATP. Muscle fibre types are characterized by their myosin isoform content and metabolic profile. For example, the type-1 fibres common in mouse soleus contain a slow myosin isoform and have a high capacity for mitochondrial/oxidative production of ATP. Disruption of the matching of contractile proteins and metabolism may result in altered contractile performance. We report here the performance of fully rested soleus (sol) and extensor digitorum longus (edl) muscles from wild-type (wt) and UCP3-oe during contraction whilst shortening. All experiments were done on muscles isolated from mice that had been killed humanely. Soleus muscles were tetanized at 75 Hz and edl at 125 Hz (25°C). The 'step and ramp' method was used to measure the force-velocity relationship of the contractile elements (Curtin et al. 1998); muscles were stimulated isometrically for 0.2 s before shortening. The slack test method (Edman, 1977) was also used to measure the velocity of shortening under zero load. Muscles from wt and UCP3-oe mice had similar Vmax values, maximum velocities of shortening (mean± s.e.; wt sol, 5.08 ± 0.23, n=11; UCP3-oe sol, 4.98 ± 0.61, n=7; wt edl, 7.84 ± 0.31, n=8; UCP3-oe edl, 8.39 ± 0.60, n=7: units Lo/s where Lo is fibre length giving maximum isometric force, Po) and similar maximum power outputs (wt sol, 0.081 ± 0.006, n=11; UCP3-oe sol, 0.074 ± 0.007, n=7; wt edl, 0.100 ± 0.002, n=8; UCP3-oe edl, 0.109 ± 0.006, n=7: units /PoVmax). We conclude that over-expression of UCP3 does not affect these aspects of the contractile performance.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

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Effect of lipopolysaccharide infusion on mRNA expression of two, muscle-specific, ubiquitin ligases in myocardium, soleus and extensor digitorum longus of the rat

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Sepsis induces skeletal muscle atrophy as a consequence of the suppression of muscle protein synthesis and stimulation of protein degradation, with proteins predominantly degraded via the ubiquitin-proteasome system (Hasselgren, 1999). Two muscle-specific ubiquitin ligases, namely, MuRF1 and MAFbx, responsible for the targeting of proteins for proteolysis, have recently been described, and their mRNA levels in fast-twitch muscle shown to increase during atrophy (Bodine et al., 2001). Here, we examined the effects of lipopolysaccharide (LPS) infusion on MAFbx and MuRF1 mRNA expression in myocardium, and muscles of a slow- and fast-twitch phenotype.

Conscious male Sprague-Dawley rats (previously implanted with jugular venous catheters under general anaesthesia (fentanyl and medetomidine, 300 $\mu g\ kg^{-1}$ each i.p.)), were divided into 6 groups and infused with either saline (0.4 ml h^{-1}) for 2h (n=8), 6h (n=7) or 24h (n=8), or LPS dissolved in saline to induce sepsis (E. coli serotype 0127:B8, 150 $\mu g\ kg^{-1}\ h^{-1}$) for 2h (n=8), 6h (n=8) or 24h (n=6). At each time point, the myocardium, slow-twitch soleus and fast-twitch extensor digitorum longus (EDL) muscles were freeze clamped in situ under terminal anaesthesia (sodium pentobarbital, i.v.) and stored at -80°C. All animal procedures complied with Home Office regulations. Total RNA was extracted from muscle samples and expression of MuRF1 and MAFbx determined by real-time PCR. The results are presented in Figure 1.

There was upregulation of MAFbx and MuRF1 mRNA in skeletal muscle within 6 hours after onset of LPS infusion. Interestingly, the increase in MAFbx was restricted to the EDL, while expression of MuRF1 showed a time-dependent increase in soleus and EDL. LPS had no effect on MAFbx or MuRF1 expression in myocardium (Figure 1). These findings are in agreement with evidence implicating a central role for MuRF1 and MAFbx in skeletal muscle atrophy of various catabolic conditions (Lecker et al. 2004).

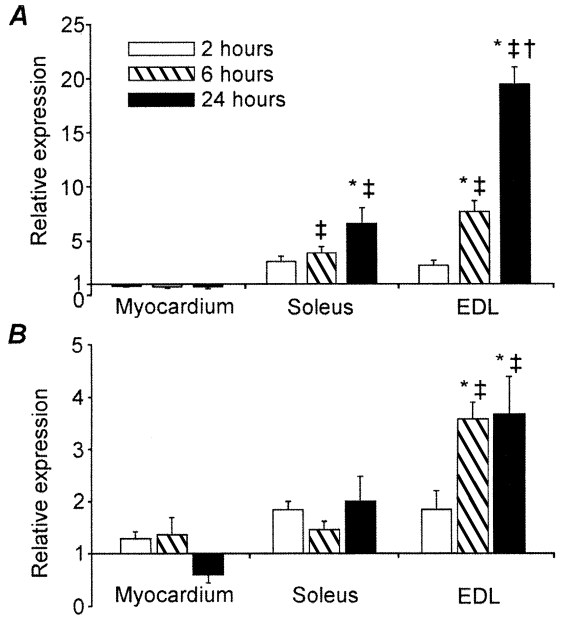

Figure 1. Fold changes in A) MuRF1 and B) MAFbx mRNA expression from corresponding control value in myocardium, soleus and EDL muscles in response to LPS. Values represent mean ± S.E. Indicates different from corresponding control (P<0.05, MANOVA); * different from 2 hour LPS-treated muscle (P<0.05, MANOVA, LSD post-hoc); different from 6 hour LPS-treated muscle (P<0.05, MANOVA, LSD post-hoc).

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

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Lipopolysaccharide infusion rapidly alters pyruvate dehydrogenase kinase mRNA expression in rat skeletal muscle

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Changes observed in skeletal muscle metabolism during sepsis are consistent with impaired oxidative metabolism, and in particular to a defect in pyruvate utilisation, due possibly to an inhibition of the pyruvate dehydrogenase complex (PDC; Vary et al. 1999). Phosphorylation (inactivation) of the PDC is regulated by pyruvate dehydrogenase kinase (PDK), of which isoforms 2 and 4 are the most prevalent in skeletal muscle. The aim of the present study was to examine the effect of lipopolysaccharide (LPS) infusion on PDK2 and PDK4 mRNA expression in fast- and slow-twitch skeletal muscle of the rat.

Male Sprague-Dawley rats (previously implanted with jugular venous catheters under general anaesthesia (fentanyl and medetomidine, 300 μg kg^{-1} each i.p.)), were divided into 6 groups, and infused with either saline (0.4 ml h^{-1}) for 2 h (n=8), 6 h (n=7) or 24 h (n=8), or LPS (*E. coli* serotype 0127:B8, dissolved in saline, 150 μg kg^{-1} h^{-1}) for 2 h (n=8), 6 h (n=8) or 24 h (n=6). At each time point, the slow-twitch soleus and fast-twitch extensor digitorum longus (EDL) muscles were freeze clamped in situ under terminal anaesthesia (sodium pentobarbital, i.v.) and stored at -80°C. All animal procedures complied with Home Office regulations. Total RNA was extracted from muscle samples and expression of PDK2 and PDK4 determined by real-time PCR. The results are presented in Table 1.

LPS infusion resulted in a marked and time-dependent increase in PDK4 mRNA expression in EDL and soleus, whereas a less marked time-dependent decrease of PDK2 expression was observed in soleus (Table 1). These findings are consistent with down regulation of carbohydrate oxidation following LPS administration, which, based on the present data, may be due to PDC inactivation as a consequence of a rapid and marked increase in PDK4 mRNA and presumably protein expression.

Table 1: Fold changes in PDK2 and PDK4 mRNA expression from the corresponding control value in EDL and soleus muscles of the rat after LPS infusion

	PDK2		PDK4	
	EDL	Soleus	EDL	Soleus
2h	1.05 ±0.13	1.10 ±0.13	3.35 ±1.03	2.26 ±0.48
6h	1.30 ±0.15	0.92 ±0.13	9.61 ±0.99 ‡	9.16 ±1.60 ‡*
24h	0.76 ± 0.16	0.68 ±0.02 ‡	23.52 ±4.43 ‡*†	7.78 ±1.39 ‡*

Values are mean ± S.E. * Significantly different from 2h LPS treated muscle (P<0.05 MANOVA, LSD post-hoc). †Significantly different from 6h LPS treated muscle (P<0.05 MANOVA, LSD post-hoc). ‡Significantly different from saline control at corresponding time point (P<0.05 MANOVA).

Vary TC et al. (1999). Mol Cell Biochem **198**, 113-118.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

C100

Distribution of atrial natriuretic peptide and its effects on contraction and Ca^{2+} in ventricular myocytes from streptozotocin-induced diabetic rat

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Atrial natriuretic peptide (ANP) is a hormone released predominantly by atrial myocytes in response to atrial wall stretch. Levels of plasma ANP are increased during experimental hypoxia and in various disease states including coronary heart failure, myocardial infarction and diabetes (Sahai & Ganguly, 1992). The distribution of ANP and its effects on contraction and intracellular Ca^{2+} in ventricular myocytes from streptozotocin (STZ)-induced diabetic rat have been investigated. Diabetes was induced in male Wistar rats (220-250 g) by a single ip. injection of STZ

(60 mg/kg body weight). Experiments were performed 8-12 weeks later after STZ treatment. Animals were killed humanely. Blood glucose in STZ-treated rats was 347.4 ± 15.2 mg/dl compared to 68.0 ± 2.3 mg/dl in age-matched controls (n=5). Immunoconfocal techniques were used to measure the distribution of ANP. STZ treatment significantly increased the number of myocytes immunolabeled with antibodies against ANP. The percentage of ANP(+) and ANP(-) labelled myocytes from control rat were 16.8 ± 5.3 % and 83.1 ± 5.3 % (n=67), respectively. However, the percentage of ANP(+) and ANP(-) labelled myocytes from STZ-treated rats were 52.1 ± 7.2 and 53.2 ± 4.6 % (n=67), respectively. Shortening was measured in electrically stimulated (1 Hz) myocytes superfused with normal Tyrode solution containing 1 mM Ca^{2+} at room temperature with a video edge detection system (VED-114, Crystal Biotech, USA). Time to peak (TPK) shortening was significantly prolonged in myocytes from STZ-treated (360 ± 5 ms, n=19) compared to controls (305 ± 5 ms, n=27). Amplitude of shortening was marginally increased in myocytes from STZ-treated rat and treatment of myocytes with $1x10^{-7}$ M ANP for 1 h produced a small reduction in amplitude of shortening in myocytes from both STZ-treated and control rats. Intracellular [Ca^{2+}] was measured in fura-2/AM loaded myocytes. The amplitude of the Ca^{2+} transient was significantly increased in myocytes from STZ-treated rats (0.39 ± 0.02, n=20 fura-2 ratio units) compared to controls (0.29 ± 0.02, n=26 fura-2 ratio units) and treatment with ANP reduced the amplitude to control levels. Our results show that the distribution and level of ANP is upregulated in diabetic rats and that ANP may have a Ca^{2+} modulatory role in ventricular myocytes from STZ-induced diabetic rat. Data are means ± S.E.M. Statistical comparisons were made using independent sample t test or ANOVA followed by Bonferroni corrected t test for multiple comparisons, as appropriate. P values less than 0.05 were considered significant. Ethical clearance obtained from the Faculty of Medicine & Health Sciences Ethics Committee, United Arab Emirates University.

Sahai A & Ganguly PK (1992) J Physiol Pharmacol 36, 3-14.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

C101

Quadriceps femoris activation and EMG median frequency after knee surgery

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Common findings after anterior cruciate ligament reconstruction (ACLR) are quadriceps muscle weakness, pain and effusion. Reduced voluntary activation may play a role, where activation is defined as an inability to recruit all motor units to a maximum level during a maximum voluntary contraction (MVC) (Rutherford et al. 1986). Determination of the median frequency of the surface electromyographic (EMG) signal has been used to estimate motor unit firing frequencies (Stulen & De Luca 1981). With ethical approval and informed consent, we examined the relationship between the median frequency of firing of surface electromyographic (EMG) recordings one and three months after surgery.

Thirty one patients (25 male (M), 6 female (F)) recovering from ACLR: mean(SD) aged 30(8) years, body mass 76(9) kg, height 1.75(0.10) m took part in this study and were tested 1 and 3 months after surgery. Five recreationally active subjects (2M, 3F) aged 28(3) years, height 1.69(0.1) m, body mass 65(11) kg acted as controls. Muscle function tests included twitch superimposition and Fast Fourier transformation of surface electromyographic (EMG) recordings (5,000 cycles per second, filter 10-250 Hz) during 5s MVC to determine median frequency of motor unit action potentials of rectus femoris at 100%, 75%, 50% and 25% of MVC. Knee pain was measured using a visual analogue scale. ANOVA and post-hoc paired t tests were used to test for differences. Results are reported as means (S.E.M.)

One month after surgery, a significant correlation (r=0.67, P<0.01, n=31) was found between the level of activation and strength of injured knee extensors. By 3 months post surgery, most patients (n=27) had achieved full activation but still had muscle weakness (ratio injured/uninjured I/U limb 61(3)%, MVC 127(10) Nm, 202(11) Nm, P<0.0001); the four patients demonstrating incomplete activation reported no pain. At 1 and 3 months post surgery, the EMG median frequency was significantly lower (P<0.05) in the injured compared to the uninjured limb except for 25% MVC (Fig 1); EMG amplitude was significantly (P<0.05) lower in the involved compared to the uninvolved quadriceps. Our results showed improvement in volitional activation of the quadriceps and lowered rates of motor unit firing 3 months after ACLR suggesting changes in patterns of activation and in the recruitment patterns of Type 11b fast contracting muscle fibres.

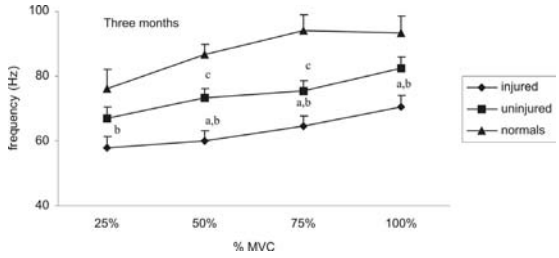

Figure 1. EMG median frequencies injured (I) and uninjured limbs (U) (n=22) three months after ACL reconstruction, and average median frequencies of limbs of 5 normal (N) subjects (n=10) Means, SEM a=p<0.05 I and U, b=p<0.05 I and N, c=p<0.05 U and N.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

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Ca^{2+} transients in skeletal muscle during repeated electrical stimulation and the effect of N-benzyl-p-toluene sulphonamide

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At the end of a long bout of activity, Ca^{2+} release from the sarcoplasmic reticulum (SR) decreases. Two mechanisms that may underlie the decline in SR Ca^{2+} release are 1. feedback of Ca^{2+} on the ryanodine receptor to decrease SR Ca^{2+} release or 2. precipitation of inorganic phosphate (Pi) and Ca^{2+} in the SR which reduces the free SR $[Ca^{2+}]$ available for release. Recently, N-benzyl-p-toluene sulphonamide (BTS) has been found to act as a potent and specific inhibitor of muscle myosin and also to have no effect on the Ca^{2+} transients associated with twitches (Cheung et al., 2002). In the presence of BTS, repeated electrical stimulation of muscle would be expected to result in decreased usage of ATP by the cross-bridges, reduced [Pi] and slower formation of Ca^{2+}-Pi precipitates in the SR. This study was approved by the Stockholm North local ethical committee. Adult NMRI mice were killed by rapid neck disarticulation. Single intact fibres were dissected from the flexor digitorum brevis, a fast toe muscle. Fibres were stimulated to contract using 70 Hz, 350 ms tetani and fatigue was induced by applying stimuli at 2 s intervals until force was down to 40% of its starting value. Isometric tetanic force and $[Ca^{2+}]_i$ (monitored with the fluorescent Ca^{2+} indicator indo-1) were measured. All experiments were performed at room temperature (24 – 26 °C). After 10 min in 10 μM BTS, force was reduced by more than 90% from 393 ± 14 to 36 ± 14 kPa (mean ± S.E.M., n = 5). However, tetanic $[Ca^{2+}]_i$ was not significantly altered being 1.35 ± 0.27 μM and 1.31 ± 0.22 μM in the absence and presence of BTS respectively. The time course of the Ca^{2+} transient was not altered by BTS. The effects of BTS were essentially reversible with force recovering to about 90% of its starting value after 20 min washout. These results emphasize that BTS blocks myosin and has no effect on SR Ca^{2+} release.

Fibres were fatigued either in the absence or presence of 10 μM BTS. Force was reduced to 40% after 61 ± 15 tetani (n = 8) and 166 ± 12 tetani (n = 9) in the absence and presence of BTS respectively. Tetanic $[Ca^{2+}]_i$ declined more rapidly in the absence of BTS than in its presence. These results suggest that in intact fibres, elevated $[Ca^{2+}]_i$ does not have adverse feedback effects on SR Ca^{2+} release. Instead the decline of tetanic $[Ca^{2+}]_i$ may be related to metabolic changes e.g. slower formation of Ca^{2+}-Pi precipitates in the SR.

Cheung A *et al.* (2002) *Nature Cell Biol* **4,** 83-88.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC128

A non-crossbridge contribution to the tension response to stretch revealed by myosin II inhibitors

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Stretch of an activated muscle causes a transient increase in tension that decays, after the stretch, to a level greater than the steady state isometric tension at the corresponding muscle length. We investigated the non-crossbridge contribution to this tension response by inhibiting active crossbridge cycling using three different myosin inhibitors, 2,3-butanedione monoxime (BDM; Bagni, et al., 2002), N-benzyl-p-toluene sulphonamide (BTS; Cheung, et al., 2002) and blebbistatin (Kovacs, et al., 2004).
Adult male rats were humanely killed with an intra-peritoneal injection (>200 mg·kg^{-1}) of an overdose of sodium pentobarbitone (Euthatal). Bundles of ~10 intact (fast) fibres (fibre length, L_0, ~2 mm) were isolated from the flexor hallucis brevis and mounted horizontally between a force transducer and a servomotor (initial sarcomere length = 2.5 μm; 20°C). The preparation was housed in a flow-through chamber perfused with physiological solution and continuously bubbled with 95% O_2 and 5% CO_2. Fibre bundles were tetanized and a ramp stretch of 5% L_0 (1 L_0 s^{-1}) applied on the tension plateau. Stimulation was maintained for a further 500 ms after the ramp where residual force enhancement (RFE; see Edman & Tsuchiya, 1996) was measured. Experiments were repeated with either 10 μM BTS (n=4), 5 mM BDM (n=1) or 10 μM blebbistatin (n=1) added to the control solution.
All myosin inhibitors markedly depressed active tension; compared to control, the mean (± SE) maximum tetanic tension decreased to 18 ± 0.02% in BTS. In preliminary experiments using BDM and blebbistatin tetanic tension decreased to 13% and <1%, respectively. The peak incremental tension during stretch also decreased, but to a lesser extent than tetanic tension; relative to control, the peak tension decreased to 61 ± 0.06% in BTS, 28% in BDM and 17% in blebbistatin. In contrast, RFE was not reduced by any of the drugs; relative to control, RFE was 157 ± 0.34% in BTS, 107% in BDM, and 92% in blebbistatin. RFE was also calculated as the residual tension determined by double exponential curve fitting to the tension decay after stretch. The residual tension, relative to the control, was 138 ± 0.24% in BTS, 96% in BDM and 99 % in blebbistatin. Whereas BDM increased the rate of tension decay after stretch, in BTS and blebbistatin the rates were decreased, suggesting that their mechanisms may differ. In all cases, however, myosin inhibitors did not decrease the RFE after stretch indicating that the underlying mechanism is not solely due to active cycling crossbridges.

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PC130

Effects of Rho-kinase inhibitors in rat and human epididymal vas deferens

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Noradrenaline (NA) elicits robust and sustained contractions of rat vas deferens in Ca^{2+}-free/EGTA medium (Ashoori & Tomita, 1983). Agents that deplete intracellular Ca^{2+} stores or block its release or protein kinase C activation do not inhibit the Ca^{2+}-free contraction (Amobi et al., 1999). In order to elucidate the excitation-contraction coupling pathway underlying the Ca^{2+}-free response and the possible role of Rho kinase-mediated Ca^{2+}sensitization, the effects of Rho-kinase inhibitors were investigated. The contribution of Ca^{2+}sensitization to Ca^{2+}-dependent contractions of both rat and human epididymal vas deferens were also evaluated. Male Sprague Dawley rats were killed humanely.
Epididymal portions of isolated rat vas deferens, longitudinal muscle strips and rings of circular muscle from human vasectomy specimens (patient consent and College ethical approval obtained) were set up for tension recordings and superfused (2.5 ml min-1) with Krebs'-bicarbonate solution (36 oC).
NA-induced Ca^{2+}-free contraction of rat vas deferens was relaxed dose-dependently by Rho kinase inhibitors, Y-27632 (0.1-10 μM in different experiments, Fig. 1A) or HA 1077 (not shown). IC50 values (mean ± S.E.M.) were, respectively, 1.08 ± 0.13 μM (n=10) and 1.75 ± 0.43 μM (n=8) respectively. Exposure to Y-27632 (0.01-10 μM in different experiments) did not change basal tone but Y-27632 (0.01-3 μM) reduced the maximum of concentration-response curves to NA (0.1-300 μM) in Ca^{2+}-free medium and Y-27632 (10 μM) abolished the response. In contrast, the NA response curve was unaffected by MLCK inhibitors, ML-9 (1-3μM) or wortmannin (1-3 μM) but the maximum was depressed by ML-9 (10 μM) or wortmannin (10 μM). Contractions of rat and human (longitudinal or circular muscle) vas deferens evoked in normal Krebs' (Ca^{2+}, 2.5 mM) by NA (100 μM) or high $[K+]_o$ (120 mM) were reliably depressed but not abolished by Y-27632 (1-10 μM). Abolition required the combination of Y-27632 (10 μM) and ML-9 (30 μM). These results suggest that Rho kinase-mediated Ca^{2+}sensitization is the major mechanism underlying NA-induced Ca^{2+}-free contraction of rat vas deferens and contributes to Ca^{2+}-dependent contractions of both rat and human vas deferens.

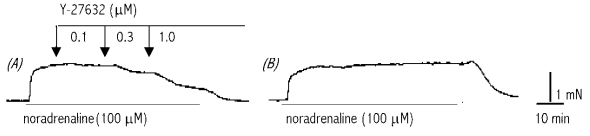

Figure 1. (A) Inhibition of NA-induced Ca^{2+}-free contraction in rat epididymal vas deferens by Y-27632 and (B) its recovery after drug washout. The tissue was equilibrated and stiumulated with NA (100 μM) in normal Krebs' medium and then superfused with Ca^{2+}-free/EGTA (1 mM) medium for 120 min before pre-contraction with NA and exposure to Y-27632. The tissue was maintained in Ca^{2+}-free/EGTA (1 mM) throughout out drug washout (90 min) and subsequent stimulation with NA.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC131

DO ADULT SKELETAL MUSCLE TRANSVERSE TUBULES CONTAIN CAVEOLA?

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Transverse tubules (TT) are plasma membrane invaginations that propagate action potentials to the inside of the skeletal muscle fiber (Melzer et al 1995). The lipid composition of isolated TT, with 35% cholesterol and 15% sphingomyelin (Anderson, 1998), is equivalent to that of caveolae and lipid rafts (Parton et al. 1997). There are conflicting reports regarding the presence of caveolin-3 in adult skeletal muscle. Some reports indicate that caveolin-3 is present in TT in developing skeletal muscle but not in adult muscle (cf. Ralston & Ploug, 1999). Other groups, however, have reported expression of caveolin-3 in TT from adult skeletal muscle using microscopy (Munoz et al. 1996) or subcellular fractionation techniques (Parton et al. 1997).

Here, we investigated the role of caveolin-3 in adult rabbit skeletal muscle fibers. New Zealand male rabbits were sedated with ketamine (50 mg/kg i.m.) and killed by cervical dislocation. By means of subcellular fractionation experiments, we found high levels of caveolin-3 in isolated TT and triads. Following a classical method to isolate rafts/caveolae (Ralston & Ploug, 1999), we isolated in sucrose density gradients light fractions from TT and triads solubilized with Triton X-100 at 4ºC. Light fractions prepared with this method were markedly enriched in caveolin-3 and cholesterol, two markers of caveolae. These results indicate that caveolin-3 is present in TT and triads isolated from adult skeletal muscle fibers, presumably forming part of cholesterol-rich caveola. Light fractions obtained from triads, however, had lower cholesterol contents and appeared at higher sucrose density than light fractions obtained from TT, suggesting that these fractions retain attached sarcoplasmic reticulum (SR) junctional proteins.

Additionally, we have analyzed the effect of cholesterol removal on the structure and integrity of isolated triads and TT. Incubation of TT with methyl-β-cyclodextrin released a fraction of the α1-DHPR to the supernatant, suggesting that cholesterol removal disrupts TT integrity.

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