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The role of myostatin and the calcineurin-signalling pathway in regulating muscle mass in response to exercise training in the rainbow trout *Oncorhynchus mykiss* Walbaum

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Summary

Rainbow trout *Oncorhynchus mykiss* Walbaum were exercised at 0.8 and 1.6 body lengths s^{-1} for 18 h a day over a 30 day period. Exercise resulted in a 24–30% increase in the average cross-sectional area of fast muscle fibres relative to tank-rested controls. The concentrations of growth factors and transcription factors hypothesised to play a role in regulating exercise-induced muscle fibre hypertrophy were measured. Exercise training resulted in a minor increase in calcineurin localisation in the nucleus. However, nuclear factor of T-cells 2 (NFAT2) nuclear localisation did not follow a pattern that was consistent with NFAT2-mediated transcriptional activity and changes in calcineurin signaling. The active peptide of myostatin, a negative regulator of muscle growth in mammals, was downregulated in exercise groups relative to tank-rested controls, but only by 6–7%. It was concluded that myostatin and calcineurin signaling do not play a major role in regulating exercise-induced muscle hypertrophy in trout.

Key words: rainbow trout, *Oncorhynchus mykiss*, exercise, muscle, hypertrophy, calcineurin, NFAT2, myostatin.

Introduction

Calcineurin is a heterodimeric Ca^{2+}/calmodulin dependent protein phosphatase that has been implicated in the regulation of skeletal muscle hypertrophy in mammals (Dunn et al., 1999; Musaro et al., 1999; Semsarian et al., 1999). Calcineurin is widely expressed in eukaryotes and highly conserved (reviewed by Guerini, 1997; Sugiura et al., 2001). Prolonged increases in intracellular Ca^{2+} levels selectively activate calcineurin, which in skeletal muscle leads to dephosphorylation of nuclear factor of T-cells 2 (NFAT2) and nuclear localisation of the calcineurin/NFAT2 complex (Shaw et al., 1995; Dolmetsch et al., 1997). The nuclear association of calcineurin and NFAT2 synergistically initiates a programme of gene expression leading to hypertrophy (Musaro et al., 1999; Semsarian et al., 1999). mRNA expression of calcineurin signaling pathway associated genes is increased in response to moderate endurance exercise training (Hitomi et al., 2003; Norrbom et al., 2004).

Myostatin or growth/differentiation factor 8 (GDF8), a member of the transforming growth factor-β (TGF-β) superfamily, is a negative regulator of skeletal muscle mass in higher vertebrates (McPherron et al., 1997). In myostatin null mice and three breeds of cattle lacking a functional myostatin protein, a hypermuscular phenotype was observed, the increase in muscle mass occurring through increased fibre number and muscle fibre hypertrophy (Kambadur et al., 1997; McPherron and Lee, 1997; Bass et al., 1999; Thomas et al., 2000; reviewed by Kocamis and Killefer, 2002). In studies on humans, myostatin mRNA expression was downregulated by 37% (Roth et al., 2003) and protein concentration was reduced by 20% (Walker et al., 2004), in response to periods of resistance training.

Forced exercise is a powerful stimulus for skeletal muscle hypertrophy in teleosts (Walker and Pull, 1973; Johnston and Moon, 1980a,b; Totland et al., 1987). Comparative studies of the structure of myostatin in teleosts and mammals indicate a high degree of sequence identity (McPherron and Lee, 1997; Roberts and Goetz, 2003). Inhibition of myostatin in transgenic zebrafish *Danio rerio* Hamilton resulted in a 20% increase in fast muscle fibre number (Xu et al., 2003). Zebrafish *D. rerio* embryos treated with a myostatin specific morpholino exhibited a marked upregulation of myogenic regulatory transcription factors MyoD and myogenin (Amali et al., 2004). These findings imply a pivotal role for myostatin during embryonic myogenesis in teleosts. In addition, myostatin mRNA expression is affected by fasting in larval and adult stages of tilapia *Oreochromis mossambicus* Peters (Rodgers et al., 2003). However, the role of myostatin in regulating muscle mass during exercise in adult fish has not been investigated.

The main aim of the present study was to use exercise training to investigate the molecular signalling pathways regulating muscle fibre hypertrophy. Specifically, we wanted to test the hypothesis that myostatin and proteins associated

with the calcineurin-signalling pathway show altered expression in response to exercise training, consistent with their having a major role in regulating muscle fibre hypertrophy, as is the case in mammals.

Materials and methods

Experimental animals

Fingerling rainbow trout *Oncorhynchus mykiss* Walbaum were obtained from College Mill Trout Farm (Almondbank, near Perth, UK) and maintained in a recirculating freshwater system (14.5–16.5°C, 14 h:10 h L:D). Fish were fed to satiation once daily (3–5% body mass M_b) with trout pellets (Trouw Aquaculture, Invergordon, UK). Juvenile mice *Mus musculus* and rats *Rattus norvegicus* were obtained from the School of Biology Animal House (University of St Andrews, UK). Atlantic salmon *Salmo salar*, approximately 4 kg M_b, were provided by Marine Harvest Scotland Ltd (Edinburgh, UK).

Experimental design

On the basis of fork length (*FL*), fish were divided into three groups of 10: TR (tank rested), E_{15} (slow endurance exercise group at 15 cm s^{-1} or 0.8 *BL* s^{-1}) and E_{30} (moderate endurance exercise group at 30 cms^{-1} or 1.6 *BL* s^{-1}). The mean *FL* of the groups was 18.4±0.4 cm (TR), 18.0±0.3 cm (E_{15}) and 18.2±0.5 cm (E_{30}) [N=10, mean ± standard error of the mean (S.E.M.)]. Initial body mass (M_b) was not measured to minimise the stress experienced by experimental animals. The exercise groups were placed into the two separate swimming channels of a purpose-built flume (Martin, 2003). The TR group was maintained in a separate 1 m diameter holding tank. Fish were acclimated to flume conditions at water flow velocities below 5 cm s^{-1}. After several days the water flow was increased gradually to 15 and 30 cm s^{-1} in either swimming channel. Fish were trained continuously for 18 h per day. Experimental animals were fed to satiation once daily during the 6 h rest period. Experimental groups had access to food for the same length of time each day. Fish were exercised for a period of 30 days. During the experiment, four fish in the E_{30} group and one fish from each of the TR and E_{15} groups were killed, because of their poor condition, by a sharp blow to the head. On day 30, all fish from the exercise experiment were killed, using an overdose of anaesthetic (MS222, tricane methanesulphonate). The groups were sampled immediately after the last period of exercise training, the fish were placed on ice after death and all tissue samples were collected within 5–30 min of death. *FL* and M_b were measured and used to calculate condition factor (CF): $CF=100(M_b/FL^3)$.

Protein extraction and SDS–PAGE

Fast muscle tissue samples (500 mg) were dissected from a region above the lateral line at the dorsal fin. Rodents were killed by a sharp blow to the head and spinal cord section, and the limb extensor muscles sectioned. Salmon *S. salar* were killed by a sharp blow to the head and fast muscle tissue samples (500 mg) were taken as in the trout *O. mykiss*. Tissue samples were placed in individual cryovials (Bibby Sterilin, Tilling Drive Stone, Staffs, UK), snap-frozen in liquid nitrogen and stored at –80°C. Total cellular protein extracts (Maniatis et al., 1989) and nuclear protein extracts (Blough et al., 1999) were prepared from individual samples. The Lowry assay determined the protein concentration in each extract (Lowry et al., 1951). For SDS–PAGE, 20 μg of total cellular protein extracts and 5 μg of nuclear protein extracts were loaded per lane. Each sample was loaded in triplicate and averaged to give a mean optical density for the concentration of a particular protein in an individual fish.

The discontinuous Laemmli system (Laemmli, 1970) was used for the electrophoresis of proteins. 6%, 12% and 15% resolving gel concentrations were used. 1 mm thick gels were cast using Mini-Protean III apparatus (Bio-Rad Laboratories Ltd., Hemel Hempstead, Herts, UK). The molecular masses of calcineurin catalytic subunit A (CnA), calcineurin regulatory subunit B (CnB) and myostatin were determined using Cruz Marker standards (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), the molecular mass of NFAT2 using high-range biotinylated standards (Bio-Rad Laboratories Ltd). Standard electrophoresis conditions were used: 100 V (constant), 30-45 mA (variable). Resolving gels were stained with Coomassie Blue (Fazekas de St Groth et al., 1963) to compare protein loading and photographed using the Versadoc 3000 imaging system (Bio-Rad Laboratories Ltd).

Western blotting

Proteins were transferred to PVDF membranes (Hybond-P, Amersham Pharmacia Biotech., Little Chalfont, Bucks, UK) using a standard technique (Towbin et al., 1979) and Mini Trans-Blot Cell (Bio-Rad Laboratories Ltd). Membranes were incubated in blocking solution (5% milk powder in PBT: phosphate-buffered saline + 0.1% Tween 20) for 1 h at room temperature (RT) to block non-specific sites. Rabbit polyclonal antibodies were screened against nuclear protein extracts from mammalian positive controls and rainbow trout (*O. mykiss*), and total protein extracts from Atlantic salmon (*S. salar*). All antibodies were diluted in blocking solution. The CnA (sc-9070 H-209, Santa Cruz Biotechnology Inc.), CnB (PC-359, Calbiochem Inc., Merck Biosciences Ltd, Nottingham, Notts, UK) and NFAT2 (sc-1149-R K18-R, Santa Cruz Biotechnology Inc.) antibodies each positively detected a single protein of the expected molecular mass (55 kDa, 19 kDa and 103 kDa, respectively) (Fig. 1A–C). Øivind Andersen (Akvaforsk, PO Box 5010, Aas, Norway) donated an antibody specific for a 16 amino acid region (347–362) of the Atlantic salmon (*S. salar*) myostatin mature peptide. The myostatin antibody positively detected three proteins: the myostatin precursor (PC, 53 kDa), latency associated peptide (LAP, 40 kDa) and myostatin mature peptide (M, 17 kDa monomer) (Fig. 1D). Primary antibodies were used at 1:500 (NFAT2), 1:1000 (CnA), 1:4000 (CnB), and 1:20,000 (myostatin). Following primary antibody incubation (4 h at RT), membranes were washed in PBT for

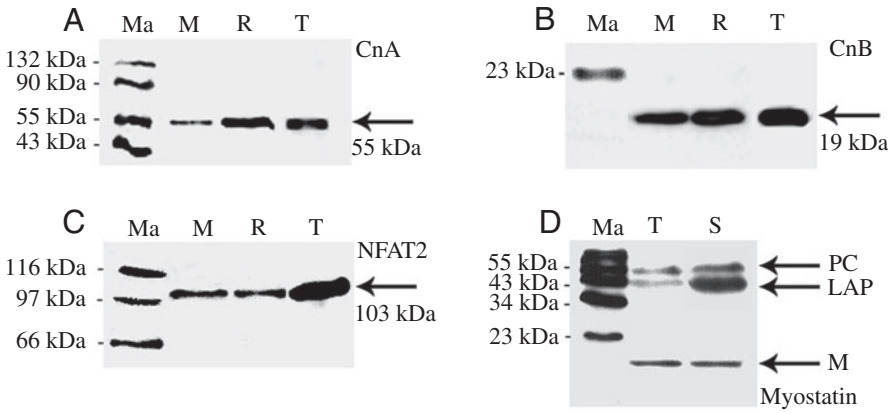

Fig. 1. Antibody screening. (A–C) The Santa Cruz human-specific CnA (sc-9070), Calbiochem mouse-specific CnB (PC-359) and Santa Cruz human-specific NFAT2 antibodies (sc-1149-R, K18-R) were screened against nuclear protein extracts. The CnA, CnB and NFAT2 antibodies each positively detected a single protein of the expected molecular mass (55 kDa, 19 kDa and 103 kDa, respectively) in the mammalian positive controls (M, mouse and R, rat) and rainbow trout (T) nuclear protein extracts. (D) Three forms of the myostatin protein were detected in total fast muscle tissue cellular extracts of the rainbow trout (T) and Atlantic salmon positive control (S): the precursor protein (PC, 53 kDa), latency-associated peptide (LAP, 40 kDa) and the mature peptide (M, 17 kDa). (A,B,D) Ma, Santa Cruz markers (sc-2035); (C) Ma, Bio-Rad high molecular mass markers (161-0311).

30 min. A horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (sc-2030, Santa Cruz Biotechnology Inc.) and ExtrAvidin HRP-conjugated secondary antibody (E2886, Sigma-Aldrich, Dorset, UK) were used at 1:1000 dilutions to detect conjugated primary antibodies and markers. Following secondary antibody incubation (1 h at RT), membranes were washed in PBT for 1 h. Proteins were visualised using the Immun-Star HRP Chemiluminescence kit (Bio-Rad Laboratories Ltd) and photographs taken using the Versadoc 3000 Imaging System (Bio-Rad Laboratories Ltd). The optical density and molecular mass of proteins were calculated using Quantity One (v. 4.4.1) image analysis software (Bio-Rad Laboratories Ltd).

Muscle fibre cellularity

A 5 mm thick steak was dissected from each fish immediately anterior to the anal fin. The steak outline was traced onto an acetate sheet for measurement of the total muscle cross-sectional area (T_{CSA}). The right hand side of the myotome was divided into four blocks of tissue. Each tissue block was placed on a cork tile, covered in cryomatrix, snap frozen in liquid nitrogen cooled isopentane (–159°C) and stored at –80°C. Transverse serial cryostat sections (7 μm) were cut at right angles to the long axis of the fish (Cryocut 1800, Reichert-Jung, Leica, Deerfield, IL, USA). Tissue sections were stained with Mayer's Haematoxylin and photographed using a frame capture camera (TK-F7300U, JVC Ltd, London, UK) connected to a light microscope with a ×10 objective (Laborlux S, Leitz, Rockleigh, NJ, USA). 1200 fast muscle cross-sectional areas (F_{CSA}) were measured per fish, from images of random fields of view, using Sigma Scan Pro image analysis software (v. 5.0.0, SPSS Inc., USA). Muscle fibre diameter (D) was expressed as the diameter of the equivalent circle from the F_{CSA} measurement: $D=2(F_{CSA}/\pi)^{0.5}$. T_{CSA} was measured using Sigma Scan Pro.

Statistical analysis

The distribution of the data was assessed using the Anderson–Darling test of normality. M_b, T_{CSA} and F_{CSA} were compared by analysis of covariance (ANCOVA) and Tukey's tests (Zar, 1996), using *FL* as the covariate. CF was analysed using a one-way analysis of variance (ANOVA) and Tukey's tests. Non-parametric smoothing and bootstrapping techniques were employed to compare distributions of muscle fibre diameters between experimental groups, using an in-house statistical program written in the open source software R (v. 1.4.1, R Foundation for Statistical Computing, Vienna, Austria). The statistical methods used are described in Johnston et al. (1999). Muscle fibre diameter distributions were compared using a non-parametric Kolmogorov–Smirnov test. If the distributions were significantly different, the 5^{th}, 10^{th}, 50^{th}, 95^{th} and 99^{th} percentiles of fibre diameter were calculated from the fitted curves and compared using a Mann–Whitney Rank Sum test (Zar, 1996).

The group mean protein concentration (± S.E.M.) was reported as the percentage of the TR group. Mean optical densities were compared using one-way ANOVA and Tukey's tests (or the non-parametric equivalents). The correlation between mean F_{CSA} and the concentration of a particular protein was assessed using a Spearman Rank Correlation. All statistical tests were performed using Minitab software (v 13.2, Minitab Inc., IA, USA) unless otherwise stated.

Results

Somatic growth

Exercised rainbow trout *O. mykiss* had a significantly greater M_b relative to *FL* (ANCOVA, N=24, P<0.001) and CF (one-way ANOVA, N=24, P<0.001) after 30 days of training in comparison with the TR group (Table 1). There was no significant difference in M_b or CF between the E_{15} and E_{30} groups. The mean T_{CSA} of the exercised groups was higher relative to the TR group (Table 1), but this increase was not significant. Overall, these data indicate that body mass accumulation was significantly augmented by exercise, but the intensity of training did not have a major effect.

Table 1. *Summary of body morphology and muscle fibre characteristics in tank-rested (TR), slow-exercised (E_{15}) and fast-exercised (E_{30}) groups of rainbow trout* O. mykiss

Experimental treatment	Swimming speed ($BL\ s^{-1}$)	Distance swum (km)	FL (cm)	M_b (g)	CF	T_{CSA} (mm^2)	F_{CSA} (μm^2)
TR	0	0	19.7±0.4	105.4±4.4	1.38±0.03	355±11	2907±64
E_{15}	0.77±0.01	292	19.4±0.3	113.8±4.8	1.55±0.04	371±11	3600±74
E_{30}	1.57±0.03	584	19.2±0.4	117.2±5.7	1.66±0.05	377±14	3769±147

Values represent group mean ± S.E.M. TR and E_{15}, N=9; E_{30}, N=6.

Fast muscle fibre growth

Compared with the TR group, mean F_{CSA} relative to FL was 24% and 30% higher in the E_{15} and E_{30} groups, respectively (Table 1; ANCOVA, N=24, P<0.001). The average probability density of muscle fibre diameter of the TR and exercised groups fell outside the 100 bootstrap estimates of the exercised and TR groups combined (Fig. 2A,B). The distributions in exercised groups were shifted to the right relative to the TR group, indicating a significant increase in fast fibre diameter in response to exercise training (Kolmogorov–Smirnov, N=24, P<0.05). Similar results were obtained for both exercised groups (Fig. 2C). The 50th, 95th and 99th percentiles of fast fibre diameter were significantly greater in the E_{15} group compared to the TR group (Mann–Whitney Rank Sum Test, N=24, P<0.01 and P<0.05, respectively; Table 2). The 5th to 99th percentiles of fast fibre diameter were significantly greater in the E_{30} group compared to the TR group (Mann–Whitney Rank Sum Test, N=24, P<0.01 and P<0.05, respectively; Table 2). These data indicate that the exercise-training regime used was sufficient to induce a marked hypertrophy of fast muscle fibres.

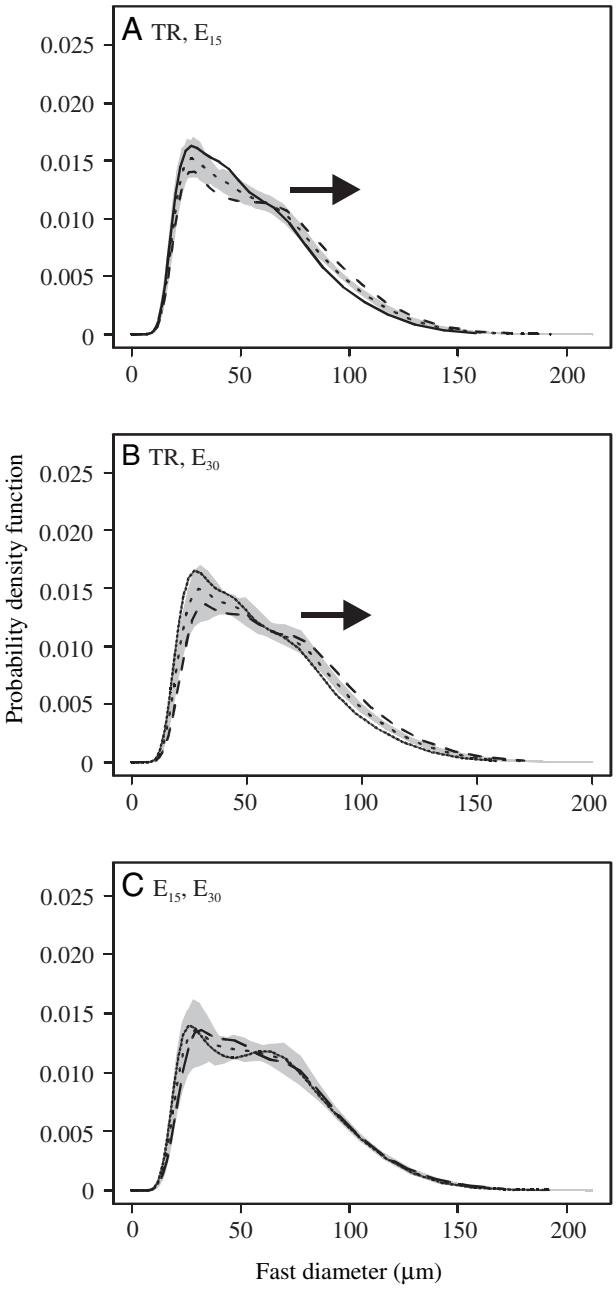

Fig. 2. The effect of tank rest (TR) or exercise training (E_{15} and E_{30}) on the distribution of fast muscle fibre diameters. The mean smooth probability density functions (pdf) of fast fibre diameter distributions for each experimental group are represented by the solid and broken lines. In each statistical comparison, the shaded area represents 100 bootstrap estimates of the fast muscle fibre diameter distribution for the two experimental groups combined. The dotted line shows the average muscle fibre distribution of the combined groups. (A) Several regions of the average probability density functions of each experimental group lay outside the grey shaded area of 100 bootstrap estimates of the total population. This suggested that there was a significant difference between the fast muscle fibre diameter distributions of the TR (solid line) and E_{15} (broken line) experimental groups (P<0.05, Kolmogorov–Smirnov). (B) Several regions of the average probability density functions of each experimental group lay outside the grey shaded area of 100 bootstrap estimates of the total population. This suggested that there was a significant difference between the fast muscle fibre diameter distributions of the TR (solid line) and E_{30} (broken line) experimental groups (P<0.05, Kolmogorov–Smirnov). (C) The average probability density functions of each experimental group lay within the grey shaded areas of 100 bootstrap estimates of the total population. This suggested that there was no significant difference in the distribution of fast muscle fibre diameters between the exercised groups (E_{15}, solid line; E_{30}, broken line). The arrows represent the apparent left-to-right shift of the distribution of fast muscle fibre diameters in E_{15} and E_{30} groups relative to the TR group. TR and E_{15}, N=9; E_{30}, N=6.

Table 2. *Percentiles of mean muscle fibre diameter (μm) for tank rested (TR), slow exercised (E_{15}) and fast exercised (E_{30}) groups*

Experimental treatment	Percentile				
	5th	10th	50th	95th	99th
TR	20.2±0.4	23.8±0.5	50.6±0.9	109.2±1.6	133.3±2.3
E_{15}	21.5±0.6	25.5±0.7	57.7±1.1**	120.5±1.2**	144.9±2.7*
TR	20.6±0.5	24.1±0.4	51.1±0.7	109.7±1.8	134.7±2.5
E_{30}	23.8±1.0**	28.4±1.5**	59.2±1.8*	121.3±1.1**	146.5±2.7*
E_{15}	21.5±0.8	25.6±1.0	58.3±1.3	119.7±1.8	143.2±3.7
E_{30}	23.8±1.0	28.4±1.5	59.2±1.8	120.8±1.9	147.9±2.7

**P<0.01, *P<0.05, Mann–Whitney rank sum test. Values represent group mean ± S.E.M. TR and E_{15}, N=9; E_{30}, N=6.

Calcineurin signaling

Nuclear localisation of CnA was 5% and 7% higher in the E_{15} and E_{30} groups, respectively, compared to the TR group (Figs 3, 4A; one-way ANOVA, N=24, P<0.001). CnB nuclear localisation was 8% higher in both exercised groups relative to the TR group (Figs 3, 4B; Kruskal–Wallis test, N=24, P<0.01). Increased CnA and CnB nuclear localisation were significantly correlated with mean F_{CSA} (Spearman Rank Correlation, N=24, P<0.001). CnA and CnB nuclear localisation was similar in the two exercised groups. The overall concentration of both calcineurin subunits in total cellular extracts was similar between groups (Figs 3, 4A,B).

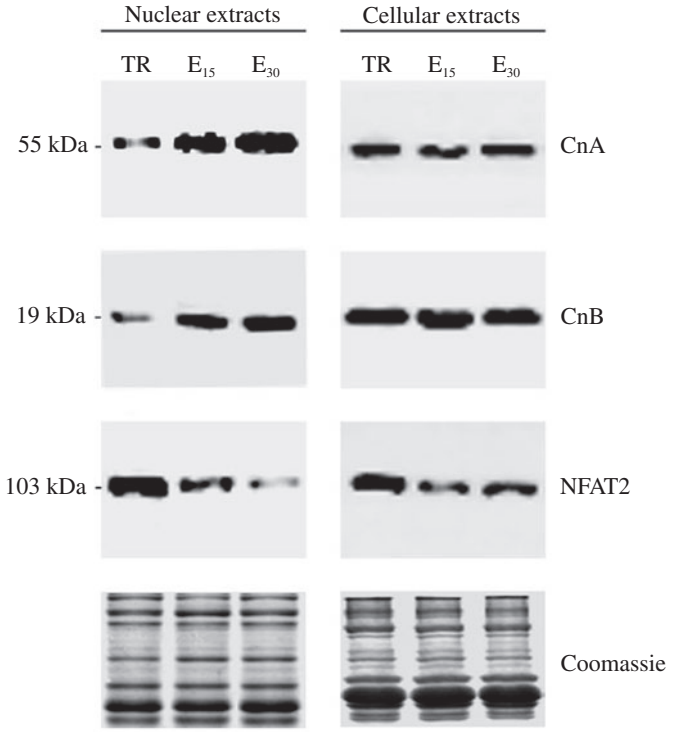

Fig. 3. The effect of tank rest or exercise training (E_{15}, E_{30}) on fast muscle tissue nuclear localisation and total cellular concentration of the calcineurin catalytic (CnA) and regulatory (CnB) subunits and associated transcription factor NFAT2. Nuclear and total cellular protein extracts were Coomassie stained to demonstrate equal sample loading. TR and E_{15}, N=9; E_{30}, N=6.

Nuclear localisation of NFAT2 was 8% and 10% higher in the TR group, relative to the E_{15} and E_{30} groups, respectively (Figs 3, 4C; one-way ANOVA, N=24, P<0.001). The nuclear localisation of NFAT2 protein was significantly inversely correlated with mean F_{CSA} (Spearman Rank Correlation, N=24, P<0.05). The overall concentration of NFAT2 in total cellular extracts was 15% and 11% higher in the TR group, relative to the E_{15} and E_{30} groups (Figs 3, 4C; one-way ANOVA, N=24, P<0.001). The overall concentration of NFAT2 was significantly negatively correlated with mean fast F_{CSA} (Spearman Rank Correlation, N=24, P<0.01). Nuclear localisation of NFAT2 and overall NFAT2 concentration were similar in the two exercised groups.

Myostatin expression

The overall concentration of the 53 kDa myostatin precursor protein (PC) was reduced by 5–9% in the E_{15} and E_{30} groups, relative to the TR group; however, the reduction was not significant. The 40 kDa latency-associated peptide (LAP) concentration was also similar between groups (Fig. 5A,B). The overall concentration of the myostatin active peptide was reduced by 6–7% in the E_{15} and E_{30} groups relative to the TR group (Fig. 5A,B) (one-way ANOVA, N=24, P<0.05). The overall myostatin active peptide concentration was inversely correlated with mean F_{CSA} (Spearman Rank Correlation, N=24, P<0.05). The concentration of the active peptide was similar in the two exercised groups.

Discussion

Exercise model of muscle fibre hypertrophy

Endurance exercise training was shown to stimulate somatic growth and muscle fibre hypertrophy in rainbow trout *O. mykiss*, as has been reported in other teleosts (Walker and Pull, 1973; Johnston and Moon, 1980a,b; Totland et al., 1987). This model of exercise-induced muscle fibre hypertrophy was used to examine potential molecular signaling pathways regulating the response.

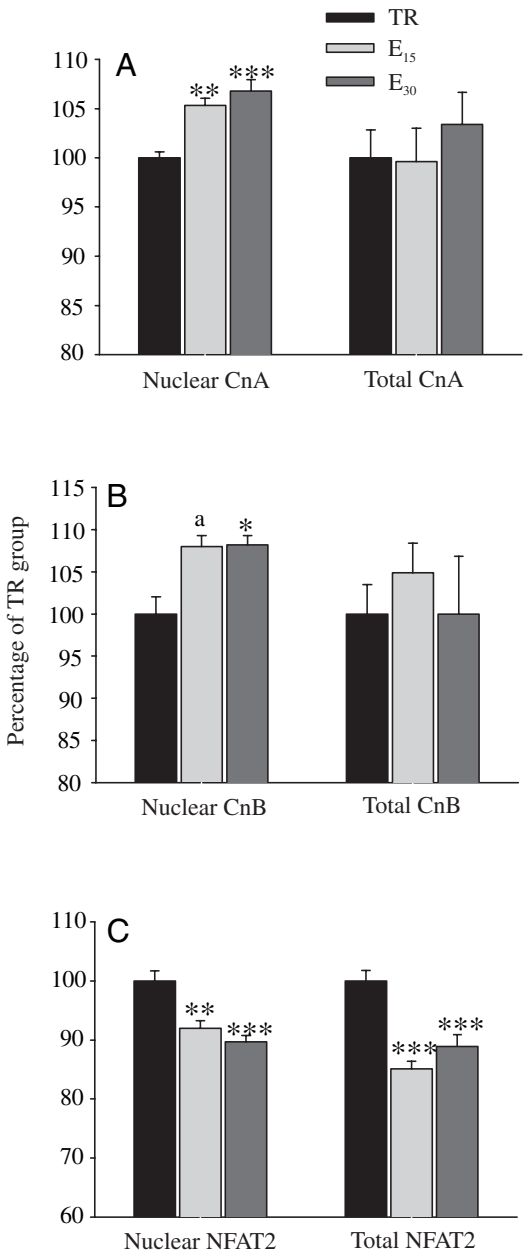

Fig. 4. (A) CnA nuclear localisation was significantly higher in the E_{15} and E_{30} groups relative to the TR group (P<0.001, one-way ANOVA; **P<0.01, ***P<0.001, Tukey's test). Overall CnA concentration in fast muscle tissue cellular extracts did not vary between the three experimental groups. (B) CnB nuclear localisation was significantly higher in the E_{15} and E_{30} groups relative to the TR group (P<0.01, Kruskal Wallis; *P<0.05, ^{a}P<0.1, *post-hoc* multiple comparisons). Overall CnB concentration in fast muscle tissue cellular extracts was invariant between the three experimental groups. (C) NFAT2 nuclear localisation was significantly reduced in the E_{15} and E_{30} groups relative to the TR group (P<0.001, one-way ANOVA; **P<0.01, ***P<0.001, Tukey's test). Overall NFAT2 concentration was significantly lower in the E_{15} and E_{30} groups relative to the TR group (P<0.001, one-way ANOVA; ***P<0.001, Tukey's test). Data are mean group optical density (± S.E.M.) expressed as a percentage of the TR group. TR and E_{15}, N=9; E_{30}, N=6.

Calcineurin – a regulator of muscle fibre hypertrophy in teleosts?

The calcineurin signaling pathway is thought to be one of several intracellular pathways in higher vertebrates that regulate hypertrophic growth of skeletal muscle (Musaro et al., 2001). *In vivo* calcineurin inhibition prevented compensatory hypertrophy of the plantaris muscle in the rat hind limb, stimulated by functional overload (Dunn et al., 1999). The co-localisation of calcineurin and NFAT2 proteins has been demonstrated in subsets of myonuclei in mammalian myofibres and the association is thought to synergistically initiate muscle-specific gene expression and muscle fibre hypertrophy (Musaro et al., 1999; Semsarian et al., 1999). In exercised rainbow trout *O. mykiss*, a slight but significant increase in calcineurin nuclear localisation was observed relative to tank-rested

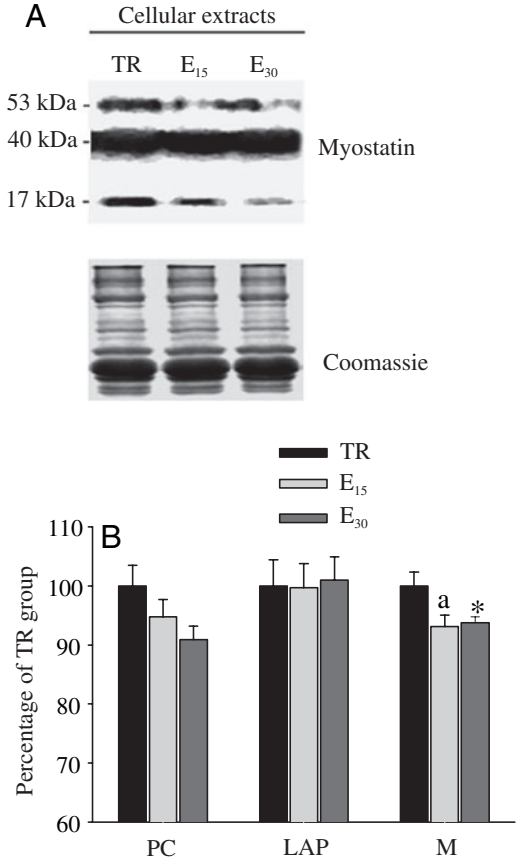

Fig. 5. (A) The effect of tank rest or exercise training (E_{15} and E_{30}) on overall myostatin concentration in fast muscle tissue extracts. (B) The myostatin PC was downregulated in the E_{15} and E_{30} groups relative to the TR group, but this was not significant. LAP concentration was also invariant between the three experimental groups. The myostatin mature peptide concentration was significantly lower in the E_{15} and E_{30} groups relative to the TR group (P<0.05, one-way ANOVA; *P<0.05, ^{a}P<0.1, Tukey's test). Fast muscle tissue total cellular protein extracts were Coomassie stained to demonstrate equal sample loading. Data are mean group optical density (± S.E.M.) expressed as a percentage of the TR group. TR and E_{15}, N=9; E_{30}, N=6.

controls. Increased calcineurin nuclear localisation was significantly positively correlated with mean F_{CSA}, which implied that calcineurin might regulate exercise-induced muscle fibre hypertrophy. However, in rainbow trout nuclear localisation of NFAT2 was not associated with exercise-induced muscle growth. Furthermore, overall NFAT2 protein concentration was markedly reduced in response to exercise, in contrast to the upregulation of NFAT2 mRNA observed with exercise in humans (Hitomi et al., 2003). A persistent non-catalytic association between calcineurin and NFAT2 is required to maintain NFAT2 in the myonuclei (Zhu and McKeon, 1999). Uncoupling of the calcineurin/NFAT2 complex or rephosphorylation of NFAT2 by vigorous kinases such as glycogen synthase kinase-3 (GSK-3) results in nuclear export and cessation of NFAT2 mediated transcription (Beals et al., 1997). These data suggest that after the initial dephosphorylation and nuclear translocation of the calcineurin/NFAT2 complex, the association between these proteins was disrupted, leading to rephosphorylation and nuclear export of NFAT2. Since nuclear localisation of the NFAT2 protein is required to mediate transcription of muscle-specific genes, these results imply that muscle fibre hypertrophy in rainbow trout *O. mykiss* is potentially calcineurin dependent, but NFAT2 independent. Interestingly, the reduced concentration of NFAT2 observed in response to exercise implies that this transcription factor may fulfil an alternative role in teleost muscle.

The present study suggests that calcineurin plays some role in the regulation of muscle fibre hypertrophy. Further work, such as the pharmacological inhibition of calcineurin through administration of cyclosporin A or FK506, or morpholino knockdown experiments targeting a calcineurin substrate such as NFAT2, could provide stronger causal evidence for the involvement of calcineurin. Until that point, however, this experiment provides an interesting insight into a potential regulatory pathway.

Myostatin – a negative regulator of muscle growth in teleosts?

An inverse relationship between muscle mass and the overall concentration of myostatin active peptide has been demonstrated in several studies with mammals (Gonzalez-Cadavid et al., 1998; Schulte and Yarasheski, 2001; McMahon et al., 2002). Moreover, myostatin expression was significantly upregulated in response to muscle damage and an atrophic stimulus (Wehling et al., 2000; Peters et al., 2003), but downregulated in response to a hypertrophic stimulus (Roth et al., 2003; Walker et al., 2004). A significant reduction of the myostatin active peptide concentration was observed in the groups of rainbow trout that displayed marked exercise-induced muscle fibre hypertrophy and this inverse relationship was significant. However, it is questionable whether this relatively small reduction, of less than one third that found in humans (Walker et al., 2004), could on its own account for the degree of exercise-induced hypertrophy observed. The double-muscled phenotype is only observed in three of the six breeds of cattle that possess a functional mutation in the myostatin protein, which suggests that dysfunction of one major gene may not entirely account for the increase in muscle mass (reviewed by Kocamis and Killefer, 2002). Similarly, inhibition of myostatin in transgenic zebrafish only resulted in a 20% increase in muscle fibre number (Xu et al., 2003).

In conclusion, it is likely that myostatin and the calcineurin signaling pathway play relatively minor roles in the intracellular signaling network regulating muscle fibre hypertrophy in this species and further work is required to elucidate the function of myostatin and calcineurin in adult fish.

List of abbreviations

BL	body length
CF	condition factor
CnA	calcineurin catalytic subunit A
CnB	calcineurin regulatory subunit B
D	muscle fibre diameter
E_{15}	slow endurance exercise group
E_{30}	moderate endurance exercise group
F_{CSA}	fast muscle fibre cross-sectional area
FL	fork length
GDF8	growth/differentiation factor 8
GSK-3	glycogen synthase kinase-3
HRP	horseradish peroxidase
LAP	myostatin latency associated peptide
M	mature processed myostatin active peptide
M_b	body mass
NFAT2	nuclear factor of activated T-cells 2
PBT	phosphate buffered saline + 0.1% Tween20
PC	myostatin precursor protein
PVDF	polyvinylidene fluoride
RT	room temperature
T_{CSA}	total fast muscle cross-sectional area
TGF-β	transforming growth factor beta
TR	tank rested

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