Regulation of expression of avian slow myosin heavy-chain isoforms

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The slow tonic anterior latissimus dorsi (ALD) muscle of the chicken contains two isomyosins, namely SM-1 and SM-2. The proportions of the two isoforms change with age, SM-2 expression increasing at the expense of SM-1. Applying a load on the wing increases the rate and extent of SM-1 replacement. Here we have demonstrated that decreasing the load by removal of the distal portion of the wing in 1-week-old chickens had an effect opposite to that of overloading in that it slowed muscle growth and the rate of SM-1 elimination. Experimental unloading of muscles previously weighted for 1 or 3 weeks slowed the growth rate of muscles, with consequent regression of relative hypertrophy; however, it did not lead to the re-expression of SM-1 myosin. This indicates that the overload-induced changes in myosin expression are not readily reversible. Nerve section produced unexpected results, in that it advanced the normal developmental shift in myosin expression toward predominance of the SM-2 isoform, similar to the effect of muscle overload.

INTRODUCTION

Growth and development of skeletal muscle are accompanied by a sequential shift in the expression of contractile and regulatory protein isoforms (Paterson & Eldridge, 1984; Butler-Browne & Whalen, 1984; Narusawa *et al.*, 1987). Such developmental shifts can fall into one of two categories: (1) the expression of an isoform can be down-regulated, allowing it eventually to be completely replaced by another isoform; this is typified during chick pectoralis-muscle development, when leg-type troponin T is replaced by its breast-type equivalent (Matsuda *et al.*, 1981); or (2) the isoforms co-exist, but their relative amounts change in a reciprocal fashion during development. The latter type of regulation is best illustrated by the developmental changes of slow myosin isoenzymes found in the chicken anterior latissimus dorsi (ALD) muscle. The ALD muscle contains two myosins, SM-1 and SM-2, which differ in the amino acid sequence of their heavy chains and are encoded by separate mRNAs (Matsuda *et al.*, 1983). Both myosins are present in the embryo as well as in the adult. In the embryo, however, SM-1 is four times as abundant as SM-2, whereas the converse is true in the adult (Hoh, 1979; Reiser *et al.*, 1988).

Many experimental interventions have been shown to retard or accelerate isoform transitions. In the case of myosin, for example, the administration of thyroid hormone induces the adult myosin isoenzyme profile precociously in rat extensor digitorum longus muscles, whereas propylthiouracil-induced hypothyroidism retards its appearance (Gambke *et al.*, 1983). Other experimental treatments, such as cross-innervation (Sal-

mons & Sreter, 1976; Hoh *et al.*, 1980), denervation (Syrový *et al.*, 1971, 1972; Gauthier & Hobbs, 1982), immobilization (Unsworth *et al.*, 1982) and work-induced hypertrophy (Gregory *et al.*, 1986) are also known to alter the myosin composition of a muscle.

Such a modulation of developmental shifts has also been demonstrated in the chicken slow tonic ALD muscle, a postural muscle responsible for keeping the chicken's wing against its body. Load-induced hypertrophy of the ALD muscle accelerates the rate and magnitude of SM-1 myosin elimination in favour of the more predominant form in the adult chicken, namely SM-2 (Kennedy *et al.*, 1986). Despite well-documented accounts of the phenotypic transitions, the stimuli that induce the changes in the expression of the two ALD-muscle myosins are unknown. In order to test whether load is responsible for the isomyosin shifts, we have examined various conditions that may affect the expression of myosin during post-hatching development of the ALD muscle.

First, we decreased the load of ALD muscle in young, rapidly growing, chickens by removing the distal portion of the wing, and we examined the progression of the isomyosin shift. Secondly, we tested whether the down-regulation of SM-1 myosin expression is permanent or whether it can be reversed when the load is removed. Finally, we attempted to determine whether the neural activity which may be associated with a given muscle load generates the stimuli affecting the myosin phenotype.

We have found that unloading growing muscles has an effect opposite to that of overloading them, in that it retards the normally occurring down-regulation of SM-1 myosin expression. Unloading the previously over-

Abbreviation used: ALD, anterior latissimus dorsi.

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loaded muscles, however, does not result in the return of SM-1 myosin expression to control levels. Load-induced changes in the myosin phenotype of the ALD muscle apparently are not readily reversible. Paradoxically, the elimination of neural activity and the induction of muscle paralysis associated with it have the same effects as does muscle overloading, indicating the complexity of the regulatory mechanisms that determine the muscle phenotype.

MATERIALS AND METHODS

White Leghorn chickens (Spafas, Norwich, CT, U.S.A.) are used in all procedures.

Unloading of the ALD

We decreased the load supported by the ALD muscle by removing the distal portion of a wing from 1-week-old chickens anaesthetized with diethyl ether. Approximately two-thirds of the humerus was removed, as well as the entire radius and ulna (approx. 5% body mass). The point of attachment of the ALD muscle to the humerus, and its nerve and blood supply, were not disturbed. The skin around the humerus and its nerve and blood supply also were not disturbed. The skin around the humerus was closed with suture (Ethicon 000 silk). The chickens received 100-125 units/g of penicillin G (potassium salt; Pfizer) for 3 days after the operation. The birds recovered quickly, eating and drinking within a few hours post-operatively, and they showed no signs of infection when they were examined 1 week later. Animals were killed by CO₂ suffocation 28, 42, or 56 days post-operatively. Control and experimental ALD muscles were removed, weighed to the nearest milligram, and processed for native polyacrylamide-gel electrophoresis. Additional, non-operated-upon, chickens were killed at the time of limb removal, and the levels of SM-1 myosin in the ALD muscle at the start of the study were recorded.

Removal of overload

A lead weight covered with gauze was wrapped around one wing of 5-week-old chickens just above the elbow. The contralateral wing was used as a control. The applied weight was maintained at 10% of the chicken's mass throughout the study. Two experimental regimens were followed; in one of these, the weight was removed 21 days after its application (long-term overload), and in the other, the weight was removed 7 days after application (short-term overload). After removal of the weight, chickens were randomly selected and killed by CO₂ suffocation at 14, 28, or 56 days. The ALD muscles were removed, weighed, and processed for native gel electrophoresis and immunofluorescence.

Denervation

The brachial plexus, which innervates the entire wing musculature, including the ALD muscle, was cut in one of the wings of 1-week-old chickens anaesthetized with diethyl ether. Regrowth was prevented by sealing the ends of the nerves within rubber tubing (Shimizu & Shimada, 1985). After this treatment, the chickens were injected daily for 3 days with approx. 125 units of penicillin G (potassium salt; Pfizer)/g as a guard against infection. To prevent wing drooping, which would cause stretch and thus additional hypertrophy of the ALD (Feng *et al.*, 1962), we attached the feathers of the

denervated wing to the chicken's body by using a self-catalysing cyanoacrylate adhesive. Birds were killed at 28 and 56 days after denervation. Control and denervated ALD muscles were excised, weighed, and processed for native gel electrophoresis. In addition, non-operated-upon control birds were killed at the time of denervation, and the level of SM-1 myosin at the start of the experiment was recorded.

Pyrophosphate (native) gel electrophoresis

ALD muscle was finely minced in 10 vol. of Guba-Straub buffer (pH 6.5)/1 mM-EDTA (d'Albis *et al.*, 1979). Electrophoresis was performed by the method of Hoh *et al.* (1976) and as described in Kennedy *et al.* (1986). After staining with Coomassie Blue R250 and destaining with 7% acetic acid, the proportions of SM-1 and SM-2 myosin in each gel were quantified. Quantification was performed with a Helena Quick Scan Jr. t.l.c. scanner equipped with a Quick Quant III integrator.

Immunofluorescence technique

We used indirect immunofluorescence with monoclonal antibodies specific for SM-1 (CCM-52), SM-2 (HPM-9), or both (HPM-7) to examine the cellular distribution of these myosins in the ALD muscle of chickens used in the long-term regression study. Details of this procedure, as well as characterization of the antibodies, are given by Kennedy *et al.* (1986).

Statistical procedures

The percentages of SM-1 myosin calculated from densitometric data were logit-transformed for normalization of the data before statistical analysis. We used a paired *t*-test to compare the percentage of SM-1 in each experimental ALD muscle with that of its contralateral control (Sokal & Rohlf, 1981). A value of *P* < 0.05 was used for determination of statistical significance.

RESULTS

Effects of reduced load

Since increased load accelerates the rate and extent of SM-1 myosin elimination, we asked whether decreasing the load would have an opposite effect. We decreased the weight of the wing by removing its distal portion. The resulting decrease in the load imposed on the ALD muscle lowered its rate of growth. The average mass of the ALD muscle 28 days post-operatively was approx. 20% less

Table 1. Relative muscle mass of ALD muscle after removal of the distal portion of one wing

Data are given as means $\pm$ S.E.M., and *n* is the number of animals used at each time point.

Time after operation (days)	<i>n</i>	Muscle mass (mg)		Difference of experimental from control (%)
		Control	Experimental	
0	10	18.7 $\pm$ 1.5	—	—
28	6	119.8 $\pm$ 6.1	98.1 $\pm$ 3.9	-17.4 $\pm$ 3.8
42	6	214.8 $\pm$ 13.2	145.4 $\pm$ 8.5	-31.9 $\pm$ 3.9
56	5	314.0 $\pm$ 17.7	220.5 $\pm$ 14.3	-29.6 $\pm$ 2.9

Fig. 1. Isomyosin transitions in the ALD muscle after removal of the distal portion of one wing

Chickens were 1 week old at the time of the operation. The nerve and blood supply to the ALD muscle were left intact. Chickens were killed at 0 ($n = 10$), 28 ($n = 6$), 42 ($n = 6$), and 56 ($n = 5$) days post-operatively ($\square$). The contralateral ALD served as control ($\square$). The relative amount of SM-1 myosin in the ALD muscle was calculated from densitometric scans of pyrophosphate gels and was expressed as a percentage of the total protein present. An asterisk indicates a significant difference from the value for the contralateral control; paired t test; $P < 0.05$.

on the experimental than on the control side (Table 1). This relative decrease in mass persisted, since the experimental ALD did not reach the weight of the control even 56 days post-operatively. Analysis of the ALD-muscle isomyosin profile revealed that a decrease in the load on the ALD muscle was associated with a significant lowering of the rate of SM-1 elimination compared with that for control muscles ($P < 0.05$; Fig. 1). The observation that both muscles showed an overall decrease in SM-1 myosin during the experiment indicates that the myosin in unloaded muscles still follows the developmental shift in isoenzymes, but at a lower rate.

Recovery from long- and short-term overload

After application of a weight overload, the ALD muscle increases in size, and the proportion of SM-1 myosin decreases rapidly (Kennedy *et al.*, 1986). We tested whether this decrease is permanent. Since the relative amount of SM-1 myosin in the ALD decreases with age (Hoh, 1979), the experiments were done on 5-week-old chickens, in which both SM-1 and SM-2 isomyosins are present in approximately equal amounts. In long-term overload, the load was kept on the wing for 3 weeks and, in short-term overload, for 1 week. In both experiments, muscles were sampled at 14, 28 and 56 days after weight removal. As can be seen in Fig. 2, both the control and the experimental muscles grew during the course of the experiments. In the 'long-term' group the growth rate (mg/day) falls below that in the controls between day 0 and 14. In the 'short-term' group the muscle mass actually falls over the first 14 days, the hypertrophy regressing to control values.

Fig. 2. Recovery from long- and short-term overload

(a) Long-term overload. Chickens had a weight applied to the wing for 21 days and were killed at 0 ($n = 5$), 14 ($n = 6$), 28 ($n = 6$) and 56 ($n = 6$) days after weight removal. All experimental values were significantly different from contralateral controls. (b) Short-term overload. Chickens had the weight applied for 7 days. The n for day 0 was 6 and for the remaining time points it was 4. The values for day 0 are those published previously (Kennedy *et al.*, 1986). An asterisk indicates a significant difference from the value for the contralateral control; paired t test; $P < 0.05$. None of the other values were significantly different. $\blacklozenge$, Experimental values; $\square$, contralateral controls. Data are given as means $\pm$ S.E.M.

Despite the difference between the two groups in the extent of hypertrophy remaining in the ALD muscle, in neither group did SM-1 myosin return to control levels. In both groups, the amount of SM-1 was decreased by the overload and remained significantly less than that in contralateral controls even at 56 days after weight removal ($P < 0.05$; Fig. 3). Immunocytochemical data substantiate this observation. SM-1 was present in a fraction of the small cells (Fig. 4), whereas every cell contained SM-2 isomyosin. After removal of the long-term overload, the SM-1 content tended to increase. The differences between values at 14, 28 and 56 days after unloading, however, were not statistically significant when compared with day 0 (the end of the overload). Control muscles in the short-term experiments had more SM-1 at each sampling time because the chickens had more SM-1 at each sampling time because the chickens were 2 weeks younger than those in the long-term experiments.

Fig. 3. Isomyosin transitions in normal and previously weighted ALD muscles

(a) Long-term overload; (b) Short-term overload. The experimental conditions are as those described in Fig. 2. The contralateral ALD muscle served as the control in each experiment. The relative amount of SM-1 myosin was calculated from densitometric scans of pyrophosphate gels and was expressed as percentage of the total protein present. An asterisk indicates a significant difference from the value for the contralateral control; paired *t* test; $P < 0.05$. ▨, Control; □, experimental.

Fig. 4. Cellular distribution of SM-1 and SM-2 isomyosins in ALD muscle of chickens after long-term overload

Chickens were killed 56 days after removal of the weight. Serial cryostat sections were stained with monoclonal antibody HPM-7 (a), HPM-9 (b), or CCM-52 (c). (d) is a phase-contrast exposure. HPM-7 reacts with both slow myosin isoforms, HPM-9 only with SM-2, and CCM-52 only with SM-1. The bar represents 397 μm.

Effects of denervation

As first shown by Feng *et al.* (1962), denervation results in hypertrophy of the ALD muscle. The fact that

the dry as well as the wet weights of the muscle increases indicates that this is a true hypertrophy [Feng *et al.*, 1962; the present study (results not shown)]. At 28 days after nerve section in the present study, denervated ALD

Fig. 5. Isomyosin transitions in normal (▨) and denervated (□) ALD muscle

The brachial plexus innervating the wing was cut in 1-week-old chickens. Chickens were killed on days 0 ($n = 6$), 28 ($n = 4$) and 56 ($n = 4$) after denervation. The contralateral ALD muscle served as the control. The proportion of SM-1 myosin was calculated from densitometric scans of pyrophosphate gels and was expressed as a percentage of the total protein present. An asterisk indicates a significant difference from the value for the contralateral control; paired t test; $P < 0.05$.

muscle had a $26\% \pm 3.9\%$ greater mass than did the contralateral controls. After 56 days of denervation, two of the chickens tested still had $29\% \pm 1.8\%$ hypertrophy of the ALD muscle, whereas the other two had muscles that were $13\% \pm 5.7\%$ smaller than the contralateral controls. Despite the difference in muscle mass, both atrophied and hypertrophied ALD muscle had significantly lower amounts of SM-1 myosin than did their contralateral controls ($P < 0.05$; Fig. 5). Therefore hypertrophy of the ALD muscle, with (Kennedy *et al.*, 1986) or without the nerve, accelerated the normal developmental decrease in SM-1 myosin.

DISCUSSION

We have previously demonstrated that the reciprocal shift in the expression of SM-1 and SM-2 isomyosins seen during normal development and growth of chicken ALD muscle is increased both in rate and in extent during overload-induced hypertrophy (Kennedy *et al.*, 1986). Here we examined the effect of unloading on myosin expression by decreasing the load experienced by the ALD muscle during its normal growth. We accomplished this by removing the distal portion of the wing in 1-week-old chickens. The decrease in the weight of the wing resulted in a lowered rate of ALD-muscle growth that was associated with substantially retarded replacement of SM-1 by SM-2 isomyosin. Thus the effect of unloading of the ALD muscle on the isomyosin shift during normal growth is opposite to the effect of overloading.

The acceleration of the developmental programme for myosin expression due to overload is permanent; that is, removal of the load from a previously overloaded wing does not result in a return of SM-1 to the level seen in the contralateral control. This was true whether the overload lasted 1 or 3 weeks. The lack of a return of SM-1

expression to control levels subsequent to load removal was supported by immunofluorescence analysis. We found that only a few fibres were positive for the SM-1 isomyosin, whereas all of the fibres reacted with the anti-(SM-2 isomyosin) antibody, a finding that confirmed the predominance of the SM-2 form. The few fibres which did express SM-1 were small and located peripherally; they may represent the expression of SM-1 in a nascent population of muscle fibres which was described previously for overloaded ALD muscle (Kennedy *et al.*, 1988).

A similar non-reversible change in the myosin phenotype was shown in studies with hypothyroid dwarf mice (Whalen *et al.*, 1985). Gene regulation of myosin in skeletal muscles thus differs from gene regulation in the ventricle, where thyroid-hormone-induced changes in myosin expression are readily reversible (Hoh *et al.*, 1977; Chizzonite & Zak, 1984; Lompre *et al.*, 1984).

To examine whether the changes in myosin expression seen during normal development or as a consequence of an altered load are due to changed contractile activity, we eliminated the neural input. After the operation, both control and denervated muscles enlarged as the animals grew, although at different rates; the denervated muscles became hypertrophied when compared with the innervated contralateral controls. As with innervated overloaded muscles, we found that the denervation-induced growth of the ALD was associated with an increase in the rate and extent of elimination of SM-1 isomyosin. The response of the slow tonic ALD muscle to denervation differs from that of twitch muscles. In contrast with fast muscles of both birds (Feng *et al.*, 1962) and mammals (Hnik, 1962), which atrophy, denervation produces substantial hypertrophy of ALD muscle that persists for several weeks (Feng *et al.*, 1962). Denervated slow-twitch muscles may exhibit hypertrophy, but compared with that of ALD muscle it is minor and lasts only a few days (Gutmann *et al.*, 1966). Moreover, in the denervated ALD muscle the normally occurring developmental shift to the SM-2 isoform is accelerated. The predominance of this isoform has been correlated with the diminution in the velocity of shortening (Reiser *et al.*, 1988). In contrast, denervated mammalian slow-twitch fibres undergo biochemical and physiological changes associated with the acquisition of fast myosin which, in adult innervated slow-twitch muscle, is present only in small amount (Hoh, 1975; Gauthier & Hobbs, 1982).

The observed acceleration of the normal developmental shift in isomyosins in the denervated ALD muscle could result from a preferential accumulation of SM-2 myosin, an increased loss of SM-1 myosin, or a combination of the two. Which of these possible effects occurs can be determined from calculations based on the known myosin content of the ALD, namely 36 mg/g of wet weight (Everett *et al.*, 1983), the percentage hypertrophy (26% at 28 days after nerve section), and the change in the relative amounts of the isomyosins (Fig. 5). The calculated increase in SM-2 (60%) and decrease in SM-1 (43%) isomyosin on day 28 after denervation are both greater than the 26% increase in muscle mass. A similar trend can be seen 56 days after the operation. Thus our data indicate that the observed change in the percentage of SM-1 myosin in the denervated ALD muscle results from an increased rate of loss of SM-1 myosin and a preferential accumulation of SM-2 myosin.

The result of our denervation experiment is unexpected in that the effect of a loss of muscular activity is similar to the effect of a functional overload. This paradoxical result is not related to the loss of a putative neurotrophic factor, since inactivity produced by blocking of the acetylcholine receptor has the same effect on myosin expression as does denervation (Connold *et al.*, 1988). It is difficult to find a common denominator in the similar isomyosin shifts due to loading and due to inactivation. It is of interest that the increased rate of growth of the ALD muscle seen during overload and after denervation is accompanied in both instances by SM-1 elimination. Considering the complex nature of gene regulation (Jones *et al.*, 1988), there may not be a common denominator in diverse growth-modifying conditions. For example, stretching has been postulated to generate a growth-regulating signal, possibly via modulation of sodium channels (Vandenburgh & Kaufman, 1981). Stretch is likely to accompany normal growth as well as weight overload. It is conceivable that other signals may produce an effect similar to that of stretch, as in the loss of neural input. For an answer to these questions, it will be necessary to define the genes coding for myosin subunits and their regulatory signals.

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