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Increased association of ribosomes with myofibrils during the skeletal-muscle hypertrophy induced either by the β-adrenoceptor agonist clenbuterol or by tenotomy

Zandra HORNE and John HESKETH*
Division of Biochemical Sciences, Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen AB2 9SB, U.K.

Ribosome distribution in skeletal-muscle myofibres was investigated by immunohistochemistry and microdensitometry by using anti-(60 S ribosomal subunit) antibodies. Administration of the β-adrenoceptor agonist clenbuterol caused an increase in the staining of the myofibrillar region with this antibody relative to that found in the subsarcolemmal cytoplasm. A similar effect was observed during hypertrophy of the plantaris muscle following severance of the tendon to the gastrocnemius. The results suggest that increased association of ribosomes with the myofibrils occurs during muscle hypertrophy.

INTRODUCTION

It has been observed that in skeletal muscle a considerable proportion of ribosomes present within the myofibre are found associated with the myofibrils (Horne & Hesketh, 1990). The functional significance of this association has not been clearly defined, but it was proposed that such ribosomes are involved in the synthesis of the myofibrillar proteins; this would suggest that the muscle structural proteins are synthesized close to the cytoplasmic sites where they are incorporated into myofibrils. Such an arrangement would decrease the logistic problems involved in the synthesis and assembly of large amounts of structural filaments (Millward, 1980).

Synthesis of myofibrillar protein would be expected to be particularly high during periods of rapid muscle growth, such as those found in the young growing animal (Waterlow *et al.*, 1978; Lewis *et al.*, 1984) and in experimentally induced muscle hypertrophy. Previous experiments have shown the proportion of ribosomes associated with the myofibrils to be increased in 14-day-old rats compared with those of 51 days of age (Horne & Hesketh, 1990), and the aim of the present work was to study the extent of association of ribosomes with the myofibrils under different physiological conditions by investigating ribosome distribution in muscle undergoing hypertrophy. The characterization of changes in ribosome distribution which may occur under such conditions is an important step towards defining the significance of myofibrillar ribosomes. Immunohistochemistry with anti-(ribosomal subunit) antibodies, combined with microdensitometry, has been used to quantify the myofibrillar and subsarcolemmal ribosomes in plantaris muscle from rats in which the plantaris had been induced to hypertrophy, either by administration of the anabolic agent clenbuterol (Reeds *et al.*, 1988) or by a compensatory workload and stretching induced by severing the tendon to the gastrocnemius (Gregory *et al.*, 1986; McMillan *et al.*, 1987).

MATERIALS AND METHODS

Materials

Anti-ribosomal antibodies were raised against rat liver 60 S ribosomal subunits and characterized by e.l.i.s.a. and immunoblotting, as previously described in detail (Horne & Hesketh, 1990); the antibodies recognize specifically five major proteins of the large subunit and do not cross-react with other muscle proteins. Biotinylated anti-rabbit IgG and streptavidin–biotinylated horseradish peroxidase complex were purchased from Amersham International, Amersham, Bucks., U.K. All other chemicals were of reagent grade and purchased from either Sigma Chemical Co. or British Drug Houses, both of Poole, Dorset, U.K.

Immunocytochemistry

Both immunohistochemistry and microdensitometry were carried out as described previously (Horne & Hesketh, 1990). Plantaris muscles were carefully dissected, pinned on to cork boards to maintain fibre length and orientation, and surrounded by OCT embedding medium (Agar Scientific Ltd., Stansted, Essex, U.K.). Muscles were then frozen in isopentane, which had been precooled in liquid N_2 (Dubowitz & Brooke, 1973). Cryostat sections (5 μm) were transferred to glass slides and fixed with acetone for 10 min. After treatment with 3% (v/v) H_2O_2 in methanol for 30 min to remove endogenous peroxidase activity, sections were incubated successively with anti-(60 S subunit) antibody (or pre-immune serum), biotinylated anti-rabbit IgG and streptavidin–peroxidase complex. Peroxidase activity was revealed by using the chromogenic substrate 3,3-diaminobenzidine, and sections were examined on a Vickers M85 microdensitometer; absorbance readings (470 nm) were taken at 15 μm intervals along the length of the fibres by using a circular mask of effective diameter 1.5 μm. Five fibres were analysed per section and six sections per muscle, and the values from one muscle were then averaged. Serial sections were stained with pre-immune serum, and absorbance values for similar fibre areas were subtracted from the values obtained with the specific serum.

Animals

Clenbuterol experiment. Male hooded Lister rats of the Rowett strain were weaned at 19 days of age and fed on stock diet (CRM nuts; Labsure, K. and K. Greff, Croydon, Surrey, U.K.) *ad libitum*. At 23 days of age they were fed on a semi-synthetic diet (PW3; Pullar & Webster, 1977) *ad libitum* for 3 days, after which time they were split into two groups: the control group continued to receive diet PW3, whereas the drug-treated group received PW3 containing 2 mg of clenbuterol/kg (Maltin *et al.*, 1986).

* To whom correspondence should be addressed.

Tenotomy experiment. Young male rats (32 days old) were subjected to tenotomy under ether anaesthesia. A small incision was made in the skin of the right leg, and the tendon to the gastrocnemius was separated carefully from those to the soleus and plantaris muscle, by using blunt forceps. The distal tendon of the right gastrocnemius was then sectioned and the skin resealed with two stitches. The animals resumed normal gait quickly, and use of the right limb was not apparently impaired by surgery. Sham operations were performed on the left legs; the legs were subjected to the same surgical procedure, but the tendon was not cut.

RESULTS

The anti-(60 S subunit) antibodies produced a specific and reproducible staining pattern with longitudinal sections of skeletal muscle; there was staining of both the subsarcolemmal cytoplasm and banding in the myofibrillar region. The staining was quantified by microdensitometry, and this did not give absolute absorbance values, but rather estimated the relative staining intensity in different regions of a section or different sections. The most meaningful way to consider the present data was therefore as the ratio between staining in the myofibrillar region and that in the subsarcolemmal. Furthermore, this also decreased variation between groups of sections stained on different occasions (in a given experiment samples from each treatment group were analysed together).

Administration of the growth promoter clenbuterol to rats led to a rapid increase in muscle weight, so that hypertrophy of the plantaris was evident after 2 days (Table 1). The timing of this effect is comparable with results from other studies with young rats (Maltin *et al.*, 1986). Labelling with the anti-(60 S subunit) antibody showed the intensity of staining of the myofibrillar region of the myofibres to be increased relative to that of the subsarcolemmal cytoplasm in muscles from the clenbuterol-fed rats (Table 1). Clenbuterol increased the ratio of myofibrillar to subsarcolemmal staining by 70–124%; the effect was evident after administration for 2 days and maintained at 7 days. At both time points there was a decrease in subsarcolemmal staining, and at 2 days this was statistically significant. Administration of clenbuterol for 7 days led to an increase (86%) in the myofibrillar staining, but this was not statistically significant.

In the tenotomy experiments, severance of the distal tendon to the gastrocnemius produced a compensatory hypertrophy of the synergistic plantaris muscle, although there was no statistically significant increase in muscle weight (Table 2). This hypertrophy was associated with a transient increase in the ratio of myofibrillar to subsarcolemmal staining with the anti-(60 S subunit) antibody (Table 2); the ratio was increased by 48% 2 days after tenotomy, but was decreased to control values by 7 days, although there

Table 1. Effect of dietary clenbuterol (2 mg/kg) on distribution of anti-ribosomal-antibody staining of fibres of plantaris muscle

Values shown are means ± S.E.M., with the numbers of animals in parentheses. Control and clenbuterol groups at the same time point were compared with a two-tailed Student's *t* test: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Treatment	Muscle wt. (mg)	Anti-ribosomal-antibody staining (A_{470})		
		Myofibrillar	Subsarcolemmal	Myofibrillar / subsarcolemmal
Control (2 days)	49.8 ± 1.2 (6)	1.18 ± 0.28 (4)	3.90 ± 0.39 (4)	0.30 ± 0.06 (4)
Clenbuterol (2 days)	55.3 ± 1.1** (6)	1.33 ± 0.26 (4)	2.22 ± 0.56* (4)	0.52 ± 0.07* (4)
Control (7 days)	73.0 ± 2.6 (6)	0.73 ± 0.19 (6)	3.71 ± 0.84 (6)	0.19 ± 0.02 (6)
Clenbuterol (7 days)	82.2 ± 3.9 (6)	1.31 ± 0.43 (6)	2.53 ± 0.08 (6)	0.52 ± 0.03*** (6)

Table 2. Effect of severance of tendon to the gastrocnemius on ribosome distribution in fibres of the synergistic plantaris muscle of the same limb

Values are means ± S.E.M., with the numbers of animals in parentheses. Sham-operated control and tenotomized groups at the same time point were compared with a two-tailed Student's *t* test: $*P < 0.05$, $***P < 0.001$.

Treatment	Muscle wt. (mg)	Anti-ribosomal-antibody staining (A_{470})		
		Myofibrillar	Subsarcolemmal	Myofibrillar / subsarcolemmal
Sham-operated control (2 days)	70.0 ± 2.1 (6)	0.99 ± 0.13 (5)	2.19 ± 0.14 (5)	0.48 ± 0.06 (5)
Tenotomized (2 days)	76.0 ± 2.5 (5)	1.04 ± 0.10 (5)	1.55 ± 0.23*** (5)	0.70 ± 0.08* (5)
Sham-operated control (7 days)	97.3 ± 4.2 (6)	0.76 ± 0.22 (4)	1.49 ± 0.53 (4)	0.47 ± 0.09 (4)
Tenotomized (7 days)	114.0 ± 9.7 (4)	0.89 ± 0.17 (4)	2.37 ± 0.45* (4)	0.39 ± 0.04 (4)

was significant increase in sarcolemmal staining at this latter time. At 2 days after tenotomy, the increase in staining ratio was largely due to a decrease in subsarcolemmal staining, but the myofibrillar staining was maintained.

DISCUSSION

The specificity of the anti-(60 S subunit) antibody is such that the immunostaining obtained with these antibodies reveals the distribution of ribosomal material within the muscle fibres (Horne & Hesketh, 1990), and previous results have shown ribosomes to be present not only in the subsarcolemmal cytoplasm but also in association with the myofibrils. The present results show that in two situations of muscle hypertrophy there is an altered staining ratio, and thus an altered distribution of ribosomal material, within the myofibre. Thus in response either to compensatory hypertrophy after tenotomy or to clenbuterol there is an increase in the concentration of ribosomes associated with the myofibrils, relative to that in the subsarcolemmal cytoplasm; the increase is greater and sustained with clenbuterol.

In the absence of detailed size measurements of either the myofibrillar and subsarcolemmal compartments or of individual fibres, it is not possible to calculate accurately the relative ribosome content of the two compartments from the relative concentrations given by the staining intensities. However, assuming the fibres to be perfectly cylindrical in shape and the subsarcolemmal cytoplasm to be contained in a thin band at the periphery of such cylinders, it is possible from simple geometric considerations to calculate the approximate relative proportions of ribosomes present in the two compartments from the ratio of the staining intensities. Control muscles have a staining ratio of 2.5 (Tables 1 and 2) and, assuming the fibres to be approx. 45 μm in diameter, with a subsarcolemmal band 1.5 μm in breadth, it can be calculated that 73% of ribosomes are present in the myofibrillar region and 27% in the subsarcolemmal cytoplasm. Although only approximate, these values demonstrate the relative importance of the myofibrillar ribosomes. Assuming that both the myofibrillar and subsarcolemmal compartments increase in size by 15% during hypertrophy, then it can be calculated from the mean staining ratio (1.7; Tables 1 and 2) that 80% of ribosomes are now present in the myofibrillar region and 20% in the subsarcolemmal cytoplasm. This calculation demonstrates that, since during hypertrophy there is enlargement of the myofibres, the maintenance of ribosome concentration in the myofibrillar region actually reflects an increase in total ribosome number in this compartment. Furthermore, the 30% decrease in subsarcolemmal staining is considerably more than the decrease which could be accounted for simply by dilution owing to enlargement of the compartment by 10–15%; this suggests that there is a redistribution of ribosomes from the subsarcolemmal to the myofibrillar compartment. In addition to redistribution, there is also a recruitment of new ribosomes, as reflected in the observed increase in total muscle RNA during hypertrophy (McMillan *et al.*, 1987; Reeds *et al.*, 1988), but it is not clear at present whether the newly synthesized ribosomes are preferentially located in the myofibrillar region.

The change in staining pattern is unlikely to be associated with the reported increase in frequency of fast oxidative glycolytic fibres induced by clenbuterol (Maltin *et al.*, 1986), since the small change in fibre type caused by clenbuterol (10%; Maltin *et al.*, 1986) would require a very large difference in ribosome distribution between fibre types to account for the considerable change in the staining ratio. The observed variability in the staining pattern for a mixed muscle such as plantaris or psoas (Horne & Hesketh, 1990; Tables 1 and 2 of the present paper) was small enough to suggest that there are not large differences in staining pattern between fibre types. Furthermore, work-induced hypertrophy induced by tenotomy, which is associated with fibre-type changes (Periasamy *et al.*, 1989) opposite to those induced by clenbuterol, caused similar alterations in ribosome distribution. Thus the fact that the ribosome redistribution occurs in two different types of muscle hypertrophy suggests that increased association of ribosomes with the myofibrils is an integral event of the hypertrophic response.

Muscle ribosome distribution has thus been shown to change in two different physiological conditions, namely hypertrophy and during development over the 14–51-day period in the rat. These differences occur under conditions where there is evidence for parallel changes in actomyosin synthesis. During muscle development there is a decrease in actomyosin synthesis (Waterlow *et al.*, 1978; Lewis *et al.*, 1984), and recent results (J. Hesketh, P. Bain, G. Campbell, G. Lobley, C. Maltin & R. Palmer, unpublished work) have shown that administration of clenbuterol increases actin and myosin synthesis in the gastrocnemius muscle. The ribosome distribution observed in skeletal muscle appears therefore to reflect the synthesis of myofibrillar proteins, and this is consistent with the hypothesis that myofibrillar ribosomes are involved with the synthesis of myofibrillar proteins. Since release of mRNA from ribonucleoprotein particles has previously been suggested to be important in the control of actomyosin synthesis (Bag & Sarkar, 1975; Roy & Sarkar, 1982; Nathanson *et al.*, 1986), redistribution of ribosomes within the myofibre could be a result of increased availability of specific mRNAs.

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