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Changes in myosin heavy chain stoichiometry in pig tracheal smooth muscle during development

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The stoichiometry of the myosin heavy chains (MHCs) has been measured in the tracheal smooth muscle of the pig after electrophoresis on SDS 4% polyacrylamide gel. The ratio of slower migrating MHC to the faster migrating MHC was 2.1 in neonates, 1.5 in young and 0.95 in old pigs ($P < 0.01$) showing that MHC composition changes with development. The unequal proportion of MHCs was not compatible with a heterodimeric arrangement of the MHCs in the native molecule as proposed earlier by Rovner et al. [(1986) Am. J. Physiol. 250, C861-870] and it is suggested that native molecules may be composed of homodimer heavy chains.

Myosin heavy chain; Developmental change; (Tracheal smooth muscle)

1. INTRODUCTION

Considerable variation exists in the maximum velocity of shortening of vertebrate smooth muscles [1]. In skeletal and cardiac muscle such a functional difference has been described and is associated, at least in part, with changes in myosin isozymes [2]. During development and hypertrophy of skeletal and cardiac muscle the changes in contractile mechanics and energetics [2,3] occur concomitantly with alterations in myosin isozyme or isoform patterns [2,4].

In smooth muscle the existence of isoforms of native myosin remains controversial [5, 6] but the heavy chains of myosin exist as two isoforms of estimated M_r 204 000 and 200 000 [6, 7]. The proportion of these two heavy chains present in a smooth muscle tissue appears to be characteristic for that tissue. In pig carotid artery, human and monkey myometrium the two heavy chains occur in equal proportion [6, 8], but in other smooth muscle tissues the proportion is unequal. In rat, rabbit and guinea pig myometrium the slower migrating heavy chain, referred to here as MHC1 greatly exceeds MHC2, whereas in rat portal vein, turkey and chicken gizzard, MHC1 is less than MHC2 [7-9]. Whether changes in the proportion of MHC1 and MHC2 occur within the same tissue during hypertrophy or development have not hitherto been described in smooth muscle tissues. We report here that the stoichiometry of the two myosin heavy chains from the pig tracheal smooth muscle changes with development.

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2. MATERIALS AND METHODS

2.1. *Animals*

Trachea from young (30-60 kg, 12-20 weeks old) and old (90-140 kg, ~30 weeks old) pigs (Large White crossed with Landrace) were obtained from the local abattoir and transported in a Krebs solution at 0°C. Within 2 h of death the tracheal smooth muscle was dissected out by removing the tunica fibrosa at the ventral aspect and connective tissue at the dorsal aspect. Neonatal pigs (7-10 kg, 3-5 weeks old, same breed) were exsanguinated following ether anaesthesia and the tracheal smooth muscle similarly dissected away. All dissecting procedures and subsequent extraction of proteins were carried out at 4°C in the cold room with the tissue kept on ice.

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2.2. *Myosin preparation*

Native myosin was extracted from the tracheal smooth muscle by chopping with arterial scissors and homogenizing in an ice-cold ground glass homogenizer for 15 s in 10 vol. of 50 mM NaH_2PO_4, 1 mM K_2H_2EGTA and 0.125 mM phenylmethylsulphonyl fluoride (PMSF) at pH 7.0 and centrifuged at 12000 × *g* for 10 min at 4°C. The pellet was resuspended in a Guba-Straub solution containing 150 mM NaH_2PO_4, 300 mM NaCl, 10 mM Na_2ATP, 1 mM K_2H_2EGTA, 0.125 mM PMSF and 1 mM 2-mercaptoethanol adjusted to pH 6.7, stirred occasionally for 1 h at 4°C, then centrifuged again. The supernatant was mixed with an equal volume of glycerol and stored at −20°C. When a second extraction of the pellet was carried out with Guba-Straub solution for a further 1 h it yielded a little more native myosin, albeit in greater dilution. This fraction was identical with the first fraction with respect to its heavy chain composition on SDS 4% polyacrylamide gels. For extraction of dissociated myosin the smooth muscle tissue was finely chopped in 10 vols SDS sample buffer containing 1% (w/v) SDS, 2.5% (v/v) 2-mercaptoethanol, 50 mM Tris buffer (pH 6.8) and 10% (v/v) glycerol, incubated in a boiling water bath for 2 min and then cooled, centrifuged and the supernatant stored at −20°C for SDS-polyacrylamide gel electrophoresis.

2.3. *SDS-polyacrylamide electrophoresis of heavy chains of myosin*

The native myosin in Guba-Straub extract was dissociated into its subunits by incubating in a boiling water bath with SDS sample buffer for 2 min. Samples were loaded on high porosity SDS-polyacrylamide gel (stacking gel: T = 3%; resolving gel: T = 4%, C = 2.6%; slab thickness = 1.5 mm), and electrophoresed at 22.5 mA/slab for 5–7 h as described in [10] and stained with 0.1% Coomassie brilliant blue in 40% methanol and 10% acetic acid.

2.4. *Immunoblotting*

The protein bands in the SDS-polyacrylamide gel were transblotted onto nitrocellulose paper [11] using a Bio-Rad apparatus for 4 h at 4°C. Transblotting was checked by staining a strip of nitrocellulose paper with Amido Black stain. The polyclonal antibodies were raised against purified myosin from chicken gizzard (smooth muscle myosin antibodies) and against human platelet myosin (non-muscle myosin antibodies) in rabbits [12]. The antibodies gave the best reaction at a protein concentration of 1–2 μg/ml and were detected using a peroxidase-conjugated second antibody (goat anti-rabbit antibodies, Institut Pasteur, Paris) by incubation with horse radish peroxidase developer (Bio-Rad).

2.5. *Densitometric quantitation*

Densitometry of the Coomassie blue stained bands on the SDS-polyacrylamide gel was performed on an LKB 2202 Ultrascan laser densitometer and absorbance of the peaks recorded on an LKB 2220 recorder with integrator. For quantitation purposes the areas under the peaks of the myosin heavy chains were measured. A range of protein loadings was applied to each gel and the MHC stoichiometry found to be the same over a 4-fold concentration range. Each lane was scanned at three different places and the areas averaged. The area was computed by the LKB 2220 by dropping a perpendicular to the horizontal baseline from the trough between the two peaks. The accuracy of these ratios was checked independently using a graphic tablet with an Apple 2 computer to measure the area. An analysis of variance was performed on the area measurements to test the significance of means from the three groups of animals.

3. RESULTS

The two heavy chains of myosin described [6], were observed when Guba-Straub or SDS extracts of pig tracheal smooth muscle was electrophoresed on SDS 4% polyacrylamide gel. The relative proportions of the two heavy chains differed between pigs from each age group (fig.1). In neonatal pigs the ratio of MHC1/MHC2 was 2.1/1, in the young pigs 1.5/1 and in the old pigs 0.95/1 (table 1). The relative proportions of heavy chains differed significantly between the three groups of animals ($P<0.01$).

The stoichiometry of the heavy chains was the same when samples of tissue were taken from several locations within the trachea of an animal. It was the same irrespective of whether myosin was extracted in the native form (Guba-Straub extract) or in the dissociated form (SDS extract of whole tissue). Identification of the two bands as heavy chains of smooth muscle myosin was achieved by

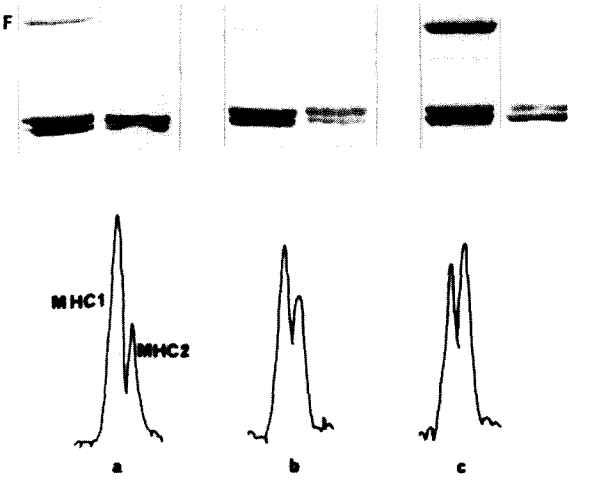

Fig.1. Heavy chains of myosin in Guba-Straub extracts of pig trachael smooth muscle revealed by SDS 4% polyacrylamide gel electrophoresis (a) neonate, (b) young and (c) old pig. Upper panels: (left) Coomassie Blue stained polyacrylamide gel section showing filamin (F) and the two myosin heavy chains MHC1 and MHC2, (right) immunoblot using antibody to smooth muscle myosin. Lower panels: densitometric scans.

Table 1

Stoichiometry of the heavy chains of myosin in tracheal smooth muscle

Group	n	Area of MHC1[a]	Ratio of MHC1/MHC2
Neonate	6	67.3 ± 1.66%	2.1 :1
Young	6	60.1 ± 1.44%	1.5 :1
Old	9	48.6 ± 1.43%	0.95:1

[a] Expressed as % of total area of MHC1 plus MHC2. Values expressed as mean ± SE

n = number of animals. Significance: neonate versus young $P<0.01$, young versus old $P<0.001$

immunoblotting using polyclonal antibodies to smooth muscle myosin where both heavy chains reacted strongly at a dilution of 1:2000 but did not react with skeletal myosin or any other proteins present on the SDS-polyacylamide gels. Non-muscle myosin could not be detected in the heavy chains using the human platelet myosin antibody at a concentration of 1:2000, at which dilution a strong positive reaction was obtained with platelet myosin electroblotted concurrently. Filamin was the other protein present in appreciable amount on the SDS-polyacrylamide gel of Guba-Straub extracts (fig.1), as shown by running a purified filamin standard from chicken gizzard along with purified standards of chicken gizzard, rabbit skeletal and bovine cardiac myosin.

4. DISCUSSION

The proportions of the two heavy chains of myosin, MHC1 and MHC2, have been determined in tracheal smooth muscle from pigs at three stages of development and found to differ considerably. The relative proportions of these heavy chains alter, such that the MHC1/MHC2 ratio declines progressively from more than 2:1 in neonates to less than 1:1 in older pigs. It is now clear that the proportion of MHC can vary greatly, e.g. from 3.5:1 in rat myometrium to 0.6:1 in turkey gizzard [7]. Whether the variety of heavy chain patterns seen [9] reflects the existence of isoforms of native myosin is not yet clear. A preponderance of one heavy chain is more likely to suggest two native myosin molecules with the heavy chains arranged as homodimers rather than a single heterodimeric native molecule [6], a proposal based on an equal proportion of heavy chains. Electrophoresis of native myosin on pyrophosphate gels has not helped to resolve this issue. Multiple bands may be seen but do not necessarily infer the presence of multiple isoforms of native myosin [5, 13], since these bands can arise from the presence of unphosphorylated, mono- or diphosphorylated myosin or filamin [14]. When these bands are excised and re-run on SDS 4% polyacrylamide gels the proportion of MHC1/MHC2 remains the same as it was in the original Guba-Straub extract or SDS extract of the tissue [8, 9]. Thus the ability of this technique to resolve isoforms of native myosin in smooth muscle cannot be assumed. In skeletal muscle, embryonic myosins cannot be resolved from adult myosins by this technique in spite of differences in the structure of their heavy chains [15].

The MHCs may arise from two different genes or from the same gene, perhaps by alternative splicing [16]. The similarity between the peptide maps of the two MHCs has not resolved this issue as yet [7]. In pig trachea no evidence for a third heavy chain of myosin was obtained which corresponded to either a non-muscle myosin [7] or a putative smooth muscle myosin reported in human airway smooth muscle [17]. The non-muscle MHC has a M_r of 196 000 and appears during culture of rat aorta [7]. Whether the shift in the relative proportion of the myosin heavy chains in tracheal smooth muscle with development is associated with functional changes in the muscle mechanics is currently being investigated.

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