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Chronic stimulation-induced effects point to a coordinated expression of carbonic anhydrase III and slow myosin heavy chain in skeletal muscle

S. Jeffery, C.D. Kelly, N. Carter, M. Kaufmann*, A. Termin* and D. Pette*

Department of Child Health, St. George's Hospital Medical School, London, UK and **Fakultät für Biologie, Universität Konstanz, D-7750 Konstanz, FRG*

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Chronic low-frequency stimulation of rat fast-twitch muscle induces 3.7-fold elevations in cytochrome *c* oxidase activity, but remains without effect on carbonic anhydrase III (CAIII) mRNA and protein. This is in contrast with the situation in the rabbit where chronic stimulation elicits more than 10-fold elevations in CAIII activity and mRNA content which coincide with an enhanced expression of the slow myosin heavy chain (HCI). Since chronic stimulation of rat muscle does not enhance the expression of HCI, we conclude that CAIII is expressed in parallel with HCI and, therefore, is present only in type I and C fibers.

Carbonic anhydrase III; Low-frequency stimulation; Muscle fiber type; Slow myosin heavy chain

1. INTRODUCTION

Carbonic anhydrase III (CAIII; EC 4.2.1.1) is one of the major soluble proteins in rat slow-twitch muscle [1]. Although there is no doubt that CAIII is found mainly in slow-twitch (type I) fibers [2], hence its high concentration in the soleus muscle, there is dispute as to whether it is a type I fiber-specific protein. A recent paper by Frémont et al. [3] has suggested that there is CAIII also in type IIA fibers. However, immunocytochemistry has shown no staining in type IIA fibers, but slight reactivity was found in fibers exhibiting myofibrillar actomyosin ATPase (mATPase) activity intermediate between fiber types IIA and IIB [4]. The incidence of these intermediate fibers is greatly increased after thyroidectomy [5].

The present study was undertaken to examine the effect of induced fiber transformation on CAIII and to address the question whether or not CAIII is a type I fiber-specific protein. For this purpose, CAIII was measured at both the mRNA and protein level in rat fast-twitch muscle subjected to low-frequency stimulation. Contrary to the rabbit, chronic low-frequency stimulation of rat fast-twitch muscle does not increase the number of type I fibers [6,7]. However, it elicits a several-fold increase in enzyme activities of aerobic-oxidative metabolism [8,9]. Therefore, measurements of cytochrome *c* oxidase (EC 1.9.3.1) activity were also undertaken.

Correspondence address: S. Jeffery, Dept. of Child Health, St. George's Hospital, Cranmer Terrace, London SW17 ORE, UK

2. MATERIALS AND METHODS

2.1. *Chronic low-frequency stimulation*

The experiments were carried out on adult male Wistar rats. Chronic low-frequency stimulation (10 Hz, 10 h daily) was performed via electrodes implanted laterally to the peroneal nerve of the left hindlimb [8]. Stimulation times, expressed in days, and number of animals (in parentheses) were: 3 (n = 3), 7 (n = 4), 14 (n = 5), 21 (n = 4), 28 (n = 6), and 56 (n = 5). The animals were killed at the respective time points and both contralateral unstimulated and stimulated tibialis anterior (TA) muscles were excised, frozen in liquid N_2 and stored at −70°C.

2.2. *Tissue extraction and cytochrome* c *oxidase assay*

Muscle pieces were pulverized under liquid N_2 and 15–20 mg of muscle powder were suspended 1:20 (w/v) in 0.1 M KH_2PO_4/Na_2HPO_4 buffer (pH 7.2) containing 2 mM EDTA. This suspension was sonicated 8 × 10 s with intense cooling and stirred on ice for 15 min. Aliquots of the homogenate were used for photometric determinations of cytochrome *c* oxidase activity at 30°C [10].

2.3. *Total RNA extraction*

The micromethod of Chomczynski and Sacchi [11] was used. Muscle powder was homogenized in 4 M guanidinium thiocyanate, 0.5% sarcosyl, 0.1 M mercaptoethanol, 25 mM sodium citrate (pH 7.0), followed by an extraction in phenol/chloroform/isoamyl alcohol. The RNA was precipitated by isopropanol and resuspended in water to a concentration of approximately 1 $\mu g/\mu l$.

2.4. *Carbonic anhydrase III measurements*

CAIII was measured by a radioimmunoassay in clarified muscle homogenate supernatants [12]. Assays were performed in triplicate at 3 dilutions using a specific antiserum to CAIII.

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2.5. *RNA slot blotting and oligo-dT hybridization*

To standardize the concentrations of mRNA used for Northern blot analysis, an oligo-dT probe was used. Total RNA preparations were applied to nylon Hybond-N membranes using a Schleicher and Schuell Minifold II apparatus [13]. Oligo-dT was end labelled using [γ-^{32}P]ATP as described by Harley [14]. The membrane was prehybridized at room temperature for 2 h in a solution containing 50% formamide, 3 × SSC, 10 × Denhardt's solution and 20 μg herring sperm DNA [15]. Hybridization was carried out at room temperature for 2 h in a solution of 50% formamide, 3 × SSC, 1 × Denhardt's solution, 20 μg herring sperm DNA, 5% dextran sulfate [15]. Four 5-min washes at room tempature in 2 × SSC were carried out.

2.6. *CAIII cDNA probe*

The cDNA probe [16] was a 1 kb fragment cut out from pUC8. The insert was radioactively labeled using [α-^{32}P]dCTP and a random hexanucleotide labeling kit (Amersham). The specific radioactivity of the cDNA probe was 6 × 10^8 dpm/μg.

2.7. *Northern blot analysis*

RNA samples were denatured in 1.1 M glyoxal, 53% DMSO, 10 mM phosphate (pH 7.0) in a total volume of 15 μl, at 50°C for 1 h. Samples were then cooled on ice. Electrophoresis was carried out in 10 mM sodium phosphate (pH 7.0) with a 1.1% agarose gel. Gels were blotted directly using 10 mM phosphate onto Hybond-N (Amersham). Filters were prehybridized overnight at 42°C using the same solutions as for the oligo-dT-labelled filters. Hybridization also involved the solution used for the oligo-dT hybridization at 42°C overnight.

2.8. *Myosin heavy chain electrophoresis*

Myosin heavy chain (HC) analysis was carried out using the previously described polyacrylamide gradient gel (5–8%) electrophoresis [6].

3. RESULTS

Time-dependent increases in the activity of cytochrome *c* oxidase were induced in the fast-twitch

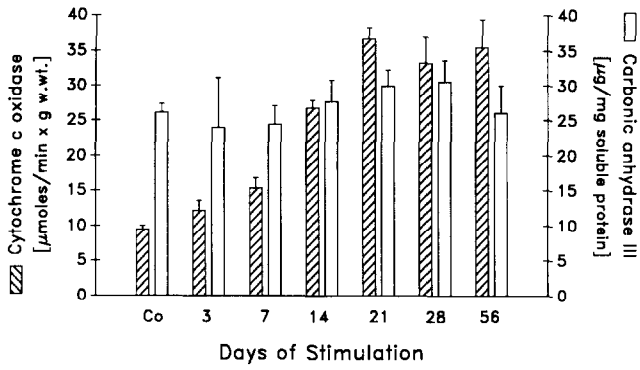

Fig.1. Time-dependent changes in cytochrome *c* oxidase activity and immunochemically assessed carbonic anhydrase III concentration in rat tibialis anterior muscle subjected to chronic low-frequency (10 Hz, 10 h daily) stimulation. The values of the unstimulated contralateral muscles of all animals were combined (Co). Values are means + SE, $n = 25$ for the contralateral muscles, and 3–5 at each time point for the stimulated muscles.

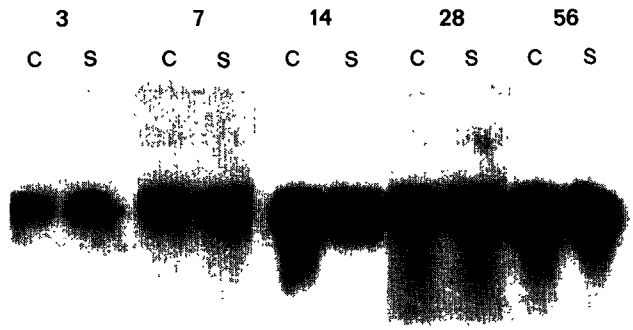

Fig.2. Northern blot analyses of RNA from control and low-frequency stimulated (10 Hz, 10 h daily) rat tibialis anterior muscles. Hybridization was carried out using a cDNA probe for rat carbonic anhydrase III. Numbers denote stimulation periods in days. C, contralateral; S, stimulated.

TA muscle during the first 3 weeks of low-frequency stimulation (fig.1). When referred to the mean value of all contralateral unstimulated TA muscles, the enzyme activity was 3.7-fold elevated after 21 days of stimulation. Prolonged stimulation did not lead to further elevations. Conversely, the immunochemically assessed low concentrations of CAIII remained unaltered in the stimulated TA muscle, even after stimulation periods of up to 56 days (fig.1). No significant differences existed between the mean values of unstimulated and stimulated TA muscles, or between the individual samples analyzed on a paired basis.

Northern blot analyses for RNA from muscles stimulated for different periods are depicted in fig.2. No differences were detected between contralateral and stimulated muscles. Equal RNA loadings were confirmed by oligo-dT hybridization on slot blots (data not shown).

In agreement with previous results on chronically stimulated fast-twitch muscles of the rat [6,7], low-frequency stimulation led to a rearrangement of the fast myosin HC isoform pattern with increases in HCIIa and HCIId at the expense of HCIIb. However, even after 56 days of stimulation there was no increase in the slow myosin HC isoform, HCI (fig.3).

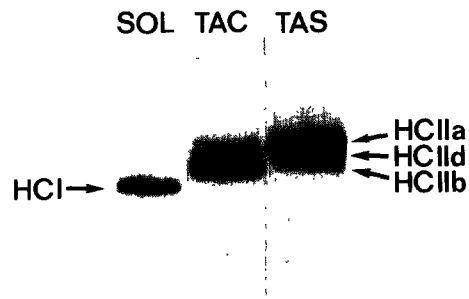

Fig. 3. Silver-stained gradient gel electrophoresis of myosin heavy chain (HC) isoforms in normal soleus (SOL), contralateral (TAC) and 56-day stimulated (TAS) tibialis anterior muscle of the rat.

4. DISCUSSION

Our results show unaltered amounts of CAIII protein and mRNA in low-frequency stimulated rat fast-twitch muscle up to stimulation periods of 56 days. Confirming previous results [8,9], stimulation clearly had an effect on rat muscle, as cytochrome *c* oxidase was increased by over 3-fold. However, the lack of changes in CAIII mRNA and protein is contrary to results from chronically stimulated rabbit TA. Gros and coworkers [17] reported that CAIII activity increased 13-fold in rabbit TA stimulated for 60 days. Increases of CAIII mRNA in the same range were observed by Brownson et al. [18] in rabbit TA after 21 days of stimulation.

The present results are easy to reconcile with species-specific differences in the response of fast-twitch muscle of small mammals to low-frequency stimulation [8,19]. The stimulation-induced changes in rat muscle remain restricted to a redistribution of fast fiber subtypes with increases in fiber types IID and IIA at the expense of type IIB [7]. At the protein level, these changes are reflected by redistributions of the fast myosin HC isoforms, HCIId, HCIIa and HCIIb. However, there are no, or only negligible, increases in myosin HCI (this study and [6,7]). Conversely, low-frequency stimulation of rabbit TA induces a progressive type II to type I fiber conversion concomitant with pronounced increases in myosin HCI at both the mRNA and protein level [19–21]. The observation that increases in myosin HCI mRNA are detectable only after 20 days of stimulation [21] is relevant in view of the results of Gros [17] who detected increases in CAIII activity only after stimulation periods of longer than 20 days. The coincidence of increases in both myosin HCI and CAIII in rabbit muscle, as well as the lack of similar changes in rat muscle, suggests a coordinated expression of these two proteins.

The existence of fibers in normal rat muscle that exhibit immunochemical reactivity for CAIII, but display mATPase staining intensities intermediate between type IIB and IIA fibers in the alkaline [4], does not invalidate the above suggestion. Although it is difficult to delineate type IIC fibers from type IIA fibers with the mATPase staining method used in that study [4], it appears likely that this small population of intermediate fibers is composed of C fibers (types IIC and IC) co-expressing fast (HCIIa) and slow (HCI) myosin heavy chain isoforms [20,22], The finding that these fibers increase by number in the hypothyroid state [5], is compatible with the enhanced expression of myosin HCI in fast-twitch muscles of the hypothyroid rat [23].

The proposed coordinated expression of CAIII and myosin HCI is in contrast with the suggestion of Frémont et al. [3] that CAIII activity also resides in type IIA fibers. Their interpretation was based on CAIII activity measurements in a muscle rich in type IIA fibers, i.e. the deep vastus lateralis muscle of the rat. However, they did not perform CAIII immunohistochemistry on mATPase-based fiber types and the CAIII activity detected [3] may be explained by type I fibers amounting to 10% of the total fiber population in that muscle [24,25].

In summary, we conclude that CAIII is expressed in parallel with the slow myosin heavy chain isoform and hence, exists in type I fibers and, at lower concentrations, also in the hybrid C fiber population co-expressing slow and fast myosins.

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REFERENCES

[1] Carter, N.D., Jefferey, S. and Shiels, A. (1982) FEBS Lett. 139, 265–266.
[2] Väänäen, H.K., Kumpulainen, T. and Korhonen, L.K. (1982) J. Histochem. Cytochem. 30, 1109–1113.
[3] Frémont, P., Charest, P.M., Cote, C. and Rogers, P. (1988) J. Histochem. Cytochem. 36, 775–782.
[4] Jeffery, S., Carter, N.D. and Smith, A. (1986) J. Histochem. Cytochem. 34, 513–516.
[5] Jeffery, S., Carter, N.D. and Smith, A. (1987) J. Histochem. Cytochem. 35, 663–668.
[6] Termin, A., Staron, R.S. and Pette, D. (1989) Histochemistry 92, 453–457.
[7] Termin, A., Staron, R.S. and Pette, D. (1989) Eur. J. Biochem. 186, 749–754.
[8] Simoneau, J.-A. and Pette, D. (1988) Pflügers Arch. Eur. J. Physiol. 412, 86–92.
[9] Hood, D.A., Zak, R. and Pette, D. (1989) Eur. J. Biochem. 179, 275–280.
[10] Wharton, D.C. and Tzagoloff, A. (1967) Methods Enzymol. 10, 245–250.
[11] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[12] Shiels, A., Jeffery, S., Wilson, C. and Carter, N.D. (1984) Biochem. J. 218, 281–284.
[13] Wahl, G. (1983) Sequences 371 (Product Sheet Schleicher & Schuell).
[14] Harley, C.B. (1987) Gene Anal. Technol. 4, 17–22.
[15] Sambrook, J., Fritsch, E.F. and Maniatis T. (1989) Molecular Cloning, 2d edn, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[16] Kelly, C.D., Carter, N.D., Jeffery, S. and Edwards, Y.H. (1988) Biosci. Rep. 8, 401–406.
[17] Gros, G. and Dodgson, S.J. (1988) Annu. Rev. Physiol. 50, 669–694.
[18] Brownson, C., Isenberg, H., Brown, W, Salmons, S. and Edwards, Y. (1988) Muscle Nerve 11, 1183–1189.
[19] Pette, D. (1990) in: The Dynamic State of Muscle Fibers (Pette, D. ed) in press, W. de Gruyter, Berlin.
[20] Staron, R.S., Gohlsch, B. and Pette, D. (1987) Pflügers Arch. Eur. J. Physiol. 408, 444–450.
[21] Kirschbaum, B.J., Heilig, A., Härtner, K.-T. and Pette, D. (1989) FEBS Lett. 243, 123–126.
[22] Staron, R.S. and Pette, D. (1986) Histochemistry 86, 19–23.
[23] Izumo, S., Nadal-Ginard, B. and Mahdavi, V. (1986) Science 231, 597–600.
[24] Armstrong, R.B. and Phelps, R.O. (1984) Am. J. Anat. 171, 259–272.
[25] Green, H.J., Klug, G.A., Reichmann, H., Seedorf, U., Wiehrer, W. and Pette, D. (1984) Pflügers Arch. Eur. J. Physiol. 400, 432–438.