Myotrophic Effects of Sciatic Nerve Extract on Carbonic Anhydrase III and Myosin Heavy Chain Expression in Myoblast Primary Cultures

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Abstract

Carbonic anhydrase III (CA III), the predominant CA isoform in skeletal muscle, is very sensitive to neuronal influences in vivo. This enzyme is rapidly up-regulated in type II muscle following denervation and this effect can be partially abolished by injections of sciatic nerve extract (SNE) in the muscle. The objectives of this study were to investigate if CA III was present in myoblasts responsible for the formation of secondary myotubes and whether its expression could still be modulated by myotrophic factor(s) present in SNE. To do so, primary myoblast cultures were established from fresh muscle biopsies of 18 day rat fetus and kept under conditions promoting myotube formation. CA III was detected very early in myoblasts but none was present in fibroblasts. Myotubes exposed to SNE during 5 days showed a 50% increase in CA III protein content when compared with control myotubes. The presence of spontaneous contractions in the myotubes exposed to SNE, in association with the analysis of the myosin heavy chain (MHC) profile, strongly suggested that SNE was promoting myotube differentiation. It is concluded that SNE can modulate the expression of CA III and MHC in muscle cells at an early developmental stage.

Key words: carbonic anhydrase, myotrophic, myoblasts, myotubes, myosin heavy chain, rat.

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Carbonic anhydrase III (CA III; E.C. 4.2.1.1.) is found at very high concentrations in the cytosol of adipocytes [19] and of mature skeletal muscle fibers [11, 30] where it is specially abundant in type I slow-twitch muscle fibers. While this enzyme has been implicated in active facilitated CO_2 transport [131 and maintenance of acid-base homeostasis, its definitive role in the muscle cell is still debated. Our interest in the recent years has been focussed mainly on clarifying the cellular function of CA III in skeletal muscle and its modulation by neuronal factors in vivo. We clearly demonstrated that denervation of skeletal muscle led to a significant and rapid increase in CA III protein content and mRNA level in type II muscle fibers [24], but this up-regulation could be prevented in part by injections of sciatic nerve extract (SNE) [23]. This therefore suggested that the apparent neuronal modulation of CA III in mature differentiated muscle fibers was at least partially related to the presence of trophic factors which favors the expression of muscle specific genes [10].

At the cellular level, it is worthwhile to note that both the CA III protein and its mRNA are expressed in 9-10 week old human foetal muscle prior to adult fiber type differentiation [17, 38]. This is an indication that CA III gene is expressed in proliferating primary myoblasts well before the formation of myotubes at the earliest stages of myogenesis and thus precedes the occurrence of nerve-muscle interactions. CA III is also found in mouse myogenic cell lines G8, C2C12 and 23A2 at different levels [39]. This observation is at variance with most myofibrillar proteins which are absent or only weakly detected in myoblasts and whose content increases mainly after myotubes formation [15]. Myotubes, which give rise to muscle fibers, arise from the fusion of at least two separate populations of myoblasts; an early population that forms the primary myotubes, and a later developing thought to be nerve-dependent population which gives rise to secondary myotubes [2, 4, 14]. In the development of mammalian skeletal muscle in vivo, slow as well as embryonic (emb) and neonatal (neo) myosin heavy chains (MHC) are expressed prior to innervation [8, 25]. However it is believed that innervation is required later on during embryonic and fetal development and during post-natal development for the

maintenance of slow MHC expression in some fibers and for the continued growth and maintenance of all muscle fibers [5, 9]. Because CA III was found in myoblasts which are responsible for the formation of primary myotubes [20], we postulated it would also be of interest to document if this ubiquitous enzyme was also expressed in secondary myoblasts. Most of these usually mature into type II fibers which have a rather low CA III content. A second objective was also to determine whether the previously demonstrated in vivo modulation of CA III by SNE could still be observed in foetal rat muscle cells put in culture at a developmental stage where they have not yet been submitted to mature neuronal influences as polyneural innervation is still present at that time [4]. Thirdly, since CA III content is expected to increase in myotubes undergoing differentiation into type I slow twitch fibers, we also wished to determine if the upregulation of CA III expression induced by SNE was correlated with the appearance of a distinct type of MHC isoform.

Materials and Methods

Sciatic nerve extract preparation

Sciatic nerves were dissected from freshly killed adult female sheep and frozen immediately. The frozen nerves were ground in liquid nitrogen with a pestle and mortar, and then homogenized in 10 mM sodium citrate buffer, pH 4.2 (40% homogenate, wt/vol). The homogenate was centrifuged for 1 hour at 20 000 X g to eliminate non soluble proteins and lipids. The soluble protein fraction was used as the sciatic nerve extract (SNE).

Myoblast cultures

Primary myoblast cultures were established from fresh muscle biopsies of 18-day Sprague-Dawley rat fetus. Myogenic satellite cells were released from minced fragments of the biopsy by serial enzyme treatments modified from Cossu et al. [7]. A first 1 hr digestion was done with 500 U/ml collagenase (Sigma Chemical Co, MO, USA) in Hank's balanced salt solution (HBSS; Gibco, NY, USA). This was followed by a 15 min incubation in HBSS containing 0.1% w/v trypsin (Gibco, NY, USA). The cell suspension was placed on uncoated 75 cm^2 Falcon plastic dishes and incubated at 37°C. After 60 min incubation, most fibroblasts (foetal fibroblast culture) became attached to the Falcon plastic dish [29]. The medium containing floating myogenic cells was collected. The myogenic cells were plated on gelatin-coated 6-well plates at a cell density of 5 X 10^4 cells/ml per well and incubated at 37°C in 5% CO_2. The culture medium (83% Dulbecco's modified Eagle's medium (DMEM; Gibco, NY, USA) containing 15% fetal bovine serum, 1% glutamine, 1% penicillin 10,000 U/ml, and streptomycin 10,000 U/ml) was changed overnight to remove cell debris. To induce myogenic differentiation, cells were moved after 2 days in a medium containing 94% DMEM, 1% glutamine, 5% horse serum. In the experimental group, 300 µg/ml of SNE was added in the medium. This dose was selected on the basis that it leads to a maximal response in terms of CA III modulation both in vivo [23] and in cell cultures [34]. The medium was changed every day and the cells were kept in culture for 5 days.

Double immunofluorescence

Myogenic cell cultures (2-day myoblasts or 5-day myotubes) were fixed in methanol at -20°C for 20 min, washed with phosphate-buffered saline (PBS), and incubated with both an anti-CA III polyclonal antibody [24] and an anti-desmin monoclonal antibody (Sigma Chemical Co, MO, USA) at the appropriate dilutions in PBS containing 0.5% BSA. After 60 min at room temperature, the cells were washed with PBS and the antigens were revealed, respectively, with goat anti-rabbit antibody coupled with tetramethyl rhodamine-isothiocyanate (TRITC) and fluorescein thiocyanate (FITC)-conjugated IgM-specific goat anti-mouse (Sigma Chemical Co, MO, USA). Fibroblast cells cultures were also fixed and incubated with both the anti-CA III polyclonal antibody and an anti-vimentin monoclonal antibody (clone 6G2) for 60 min at room temperature. The antigens were revealed, respectively, with TRITC-labelled goat anti-rabbit antibody and FITC-conjugated IgM-specific goat anti-mouse. Preparations were observed on a Zeiss Axiophot photomicroscope equipped with appropriate filter combinations.

Electrophoresis and immunoblotting

Myotubes were collected by centrifugation and homogenized exactly as described by Ho-Kim et al. [16]. The cell pellets were weighed and placed in 10 volumes of homogenizing buffer (10 mM Tris, pH 8.0, 1.0 mM EDTA, 3.3% sodium dodecyl sulfate (SDS), 10% glycerol and 40 mM dithiothreitol (DTT)) supplemented with a combination of protease inhibitors (17 µg /ml each of leupeptin, antipain-dihydrochloride and pepstatin). The homogenization was carried out using glass tissue grinders (Kontes Glass Co., N.J., USA). The SDS sample buffer was preheated to ~ 90°C and the homogenate intermittently placed in boiling water during the homogenization. The total heating time of the homogenate never exceeded 2 min. When no visible pieces of tissue were seen, the resulting homogenate was centrifuged at 10,000 g for 20 min at room temperature. The supernatant was divided into aliquots and stored at -80°C. The total protein content in the homogenates was determined by the filter paper dye-binding technique [26]. Total protein extract from fibroblasts, control myotubes (10 µg) and myotubes cultured with SNE (10 µg) were resolved on 12% SDS-polyacrylamide gels [18] and transferred to nitrocellulose [37]. The nitrocellulose sheets were incubated with an anti-CA III polyclonal antibody and with an anti-α-tubulin monoclonal antibody (Sigma), 60 min, at room temperature. The antigen-antibody complex was detected using respectively, alkaline phosphatase conjugated goat anti-rabbit (Bio/Can scientific, Mississauga, Ont, Canada) and alkaline phosphatase conjugated donkey anti-mouse (Bio/Can scientific, Mississauga, Ont, Canada). Detection of α-tubulin was used as

an internal standard to control for variations in protein loading since this protein is not influenced by the experimental treatment [23]. Myosin heavy chain isoforms (MHC) of total protein extracts from adult soleus (SOL) (2 µg), control myotubes (10 µg) and myotubes cultured with SNE (10 µg) were separated on 7-15% gradient polyacrylamide gel and stained with Coomassie blue. Protein quantification on gels and membranes was performed by image analysis using program GL-1000 of the Research Analysis System (RAS, Loats Associates, Amersham, UK).

Results

CA III is found in myoblast primary cultures

Foetal muscle cells placed in culture are able to undergo several steps of myogenic differentiation and can produce multinucleated myotubes. Double-labelling experiments performed on myoblasts in culture led to the clear colocalization of desmin (Fig. 1a, c) and CA III (Fig. 1b, d) in myoblasts. Desmin is an intermediate filament protein (IF) which gradually replaces vimentin which is present initially in myoblasts during the differentiation process. Both desmin and vimentin are associated with Z-lines during and after the assembly of the sarcomeres [12, 36]. Fibroblasts, which are the main contaminant in myoblast primary culture, do not possess desmin but instead retain vimentin, the IF protein found in all mesenchymal cell derivatives [6]. This characteristic was specially useful to differentiate fibroblasts and myoblasts in culture. After two days in culture (Fig. 1a), desmin was found in myoblasts and at the same time CA III was found to be present in the cytoplasm (Fig. 1b). Desmin (Fig. 1c) and CA III (Fig. 1d) were also found in myotubes after five days in culture. However, while fibroblasts were positive for vimentin (Fig. 1e), CA III was absolutely undetectable in these cells (Fig. 1f).

CA III protein content in primary cultures of myotubes exposed to SNE

The effect of SNE on CA III protein content in myotubes after five days in culture is presented in Fig. 2. Soluble protein extracts of control and SNE treated myotubes were analysed by Western blot using an antibody specific to CA III. Each band obtained by immunoblotting for the CA III protein was quantified by optical densitometry and the variations in CA III protein content were normalized using the α-tubulin band as an internal control. As shown in Fig. 2, the relative level of CA III protein content was significantly increased by 50% in myotubes treated with SNE (MYOT + SNE) compared with control myotubes (MYOT). CA III protein was not detected by immunoblotting in fibroblast soluble protein extracts which confirmed the immunofluorescence data.

MHC modulation by SNE in myotube primary culture

The expression of MHC isoforms was investigated in myotubes as well as in total extract of proteins from the SOL muscle of adult rat. The latter extract was added to identify MHC I and IIa [1] in order to establish a reference profile for MHC in myotubes, which in 18 day old rat fetus express only embryonic (MHCemb) and neonatal (MHCneo) isoforms [32]. As illustrated in Fig. 3, MHC_{emb}, which is known to have an electrophoretic mobility similar to MHC IIa [33], represents the major MHC isoform in control myotubes (MYOT). MHC_{neo} which has a lower electrophoretic mobility than MHC I (see SOL) significantly increases in treated myotubes (MYOT + SNE) while the amount of MHC_{emb} decreases in comparison with MYOT. These variations lead to a ratio of MHC_{emb}: MHC_{neo}, of 1.64 in MYOT, as opposed to a value of 0.31 in the MYOT + SNE (Fig. 4). In order to confirm that the MHC isoform that was upregulated in presence of SNE was indeed the MHC_{neo} and not the MHC I isoform, immunoblotting experiments were performed with an antibody specific to MHC I and the results clearly showed that MHC I was not present in these myotubes.

Discussion

This study illustrates that CA III, which is expressed at high levels in slow twitch adult rat muscles, is also observed by immunocytochemistry in primary cultures of 18-day foetal myogenic cells and measured by western analysis in myotubes. These myogenic cells are most likely secondary myoblasts since it is common belief that primary myotubes have all formed by day 16 in rat hindlimb muscle [14]. CA III was therefore immunodetected prior to and after the formation of myotubes, but absent in foetal fibroblasts. The presence of CA III in these differentiating myotubes therefore reflects an intrinsic property of this population of myoblasts from which the myotubes arose in expressing that protein even in the total absence of any neuronal component. While it is tempting to propose that, in presence of SNE, myotubes in culture were maturing toward a type II phenotype based on the fact that MHC_{neo} was becoming the predominant MHC isoform (while at the same time MHC I remained undetected), one must keep in mind that the ratio MHC_{emb}: MHC_{neo} also reflects a general state of myotube differentiation and that, under in vitro conditions, this differentiation state is also influenced by variables such as the time after obtention of myoblasts and the type of culture medium used.

Our results demonstrate that SNE can significantly influence the expression of CA III in hindlimb secondary myoblasts. The culture medium used in the present study could be lacking, as suspected by Zahradka et al [42], numerous specific growth factors. It was also shown by this group that myotube extracts display a reduced capacity to transcribe the rRNA gene because of a depletion of transcriptional factors. Assuming that synthesis of ribosomal components is coordinated in myoblasts, as shown by the basal production of CA III and of MHC isoforms, the balanced production of proteins in myotubes appears largely influenced by SNE. Soluble extracts of nervous tissue [21, 27, 28] as well as those of macrophages [3] are known to exert myotrophic or myogenic effects on skeletal muscle cells in culture and on organ culture [31, 41]. Markelonis and Oh [21, 22] have clearly shown that sciatin stimulates the morphological differentiation and the ma-

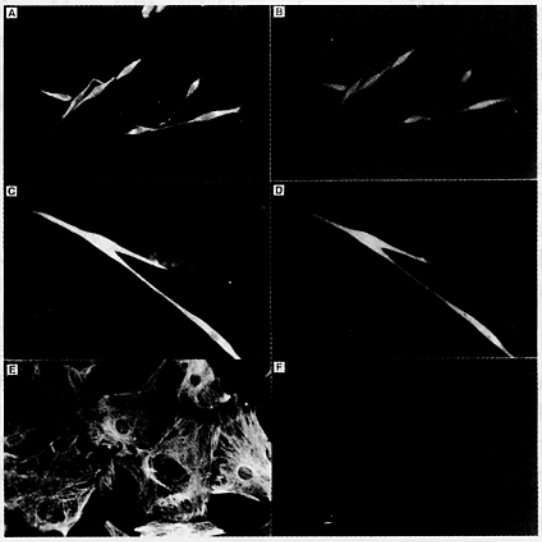

Figure 1. CA III protein detection in myoblast and myotube primary cultures. Double indirect immunofluorescence labelling of a myoblast culture after 2 days (a, b), of a typical myotube culture after 5 days (c, d) and of fibroblasts (e, f) in culture. Anti-CA III, anti-desmin and anti-vimentin were used as described in Material and Methods to reveal CA III (b, d, f), desmin (a, c) and vimentin (e), respectively.

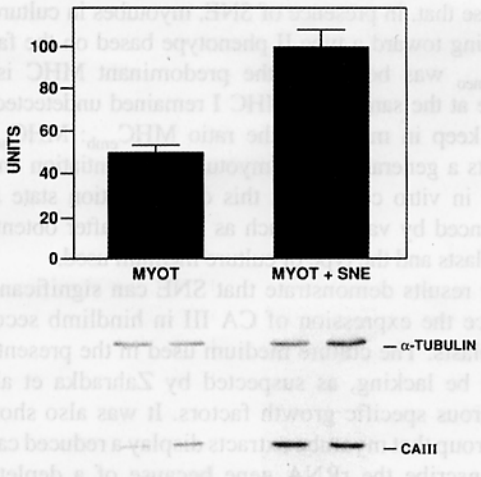

Figure 2. Quantification of CA III protein content in myotubes cultured with SNE. Each individual CA III band on the immunoblots was quantified by densitometry and normalized for the corresponding signal obtained with the α-tubulin antibody. Abbreviations: control myotubes (MYOT); myotubes cultured with SNE (MYOT+SNE). N = 6 for each group.

turation of myoblasts in culture. Except for sciatin, few proteins present in SNE have been characterized. Other trophic factors such as basic fibroblast growth factor (bFGE), ciliary neurotrophic factor (CNTF) and nerve growth factors (NGF) have been identified in SNE but their action is usually believed to be oriented mainly towards neuronal survival as they appear to be necessary for the maintenance and functioning of neural circuits [35]. Our

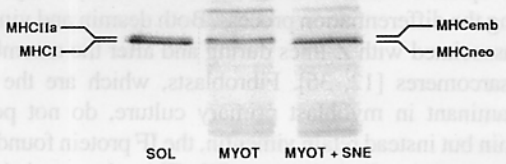

Figure 3. Separation of myosin heavy chain (MHC) isoforms by gradient gel electrophoresis of total protein extract from SOL, control myotubes (MYOT) and myotubes cultured with SNE (MYOT+SNE). Denatured proteins from total protein fraction were resolved on 7-15% gradient SDS-polyacrylamide gel and stained with Coomassie blue.

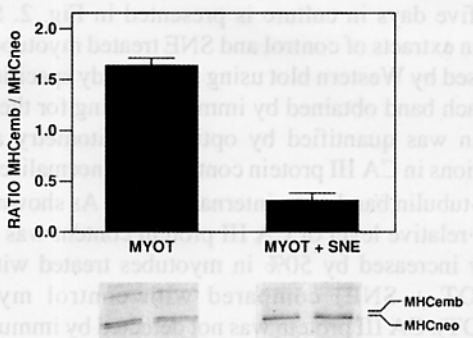

Figure 4. Value for the ratio between MHC_{emb}: MHC_{neo} in control myotubes (MYOT) and myotubes cultured with SNE (MYOT+SNE). Each individual densitometry value obtained for both isoforms was used in the determination of the mean value for the ratio MHC_{emb}: MHC_{neo}. N = 6 for each group.

own preparation of SNE [23] had certainly the capacity to promote the maturation of the overall population of myotubes under study, because they showed sustained and spontaneous contractions after 4-5 days in culture, as opposed to the quiescent state of control myotubes. Moreover, MHC_{neo} became the dominant form of MHC at the expense of MHC_{emb}, which can also be taken as a sign of maturation. Almost similar results have been observed with E19 myoblasts from late stages of development in chicken [40]. In vivo, secondary myotubes undergo the same change in their MHC isoform profile just before birth. In parallel to these signs of myotube maturation, gene machinery was obviously reactivated as evidenced by the increased production of CA III. It is therefore possible that many trophic factors thought to be only neurotrophic could also have a myotrophic influence.

At this stage, it is tempting to speculate that our SNE extract contained a transcriptional factor which was specific for the CA gene. However it is also possible that the increased expression of CA III in presence of SNE may be totally or partially associated with the SNE-induced acceleration of myotube maturation. CA III is found in both type I and type IIa fibers, even in young rat hindlimb muscles and roughly undetectable in IIb fibers. Even-though one cannot directly transpose the in vivo scheme of muscle maturation to myoblasts in culture, the fact remains that, unless the myoblasts in culture were all destined to become type IIb fibers, which is a very unlikely proposition, one would expect that myotube maturation should be accompanied by an increased CA III expression. Further experiments with purified components found in SNE would help in clarifying this issue.

In summary, CA_{III} appears to be an early marker of skeletal muscle tissue as it is detected in myoblasts, and not in fibroblasts, before and after the formation of myotubes at a stage where nerve-muscle communications are still immature. While the expression of CA III in early myoblasts seems to be an intrinsic property of these cells, the enzyme is very significantly influenced by the molecular components of SNE which also affect the overall rate of cellular maturation in vitro.

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