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AChE mRNA STABILITY IN MAMMALIAN SKELETAL MUSCLE, STUDIED *IN VITRO*

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Acetylcholinesterase (AChE) mRNA in fast rat skeletal muscle is downregulated by both, electromechanical activity and denervation. One candidate mechanism that could explain decreased level of AChE mRNA in the denervated muscle is increased rate of its degradation. In order to test this possibility, total deproteinated RNA was isolated from rat m. SM and exposed to subcellular muscular fractions prepared from contralateral m.SM. After selected time intervals, we determined remaining AChE mRNA by nonradioactive Northern blot analysis.

AChE mRNA remained at the same level during first 5 hours after denervation and abruptly fell after subsequent 13h. Further decrease in the transcript level proceeded at much slower rate. Longer transcript (3.5 kb) was more affected than the shorter (2.3 kb) one. The level of α-actin mRNA was also decreased in the denervated muscle, and the rate of its disappearance was similar to that of AChE mRNA, suggesting that AChE mRNA is not specifically affected under such conditions. Degradation of AChE mRNA was observed in all subcellular fractions studied. Postmitochondrial and postpolysomal fractions exibited higher rate than polysomal fraction. We find experimental approach demonstrated here suitable for studies of degradation capacities of the specific mRNAs in the adult skeletal muscles. Our preliminary results suggest, that fall of AChE mRNA after denervation at least partly results from increased degradation of transcripts under such conditions.

INTRODUCTION

Most skeletal muscle genes are expressed at similar levels in electrically active, innervated muscle, and in electrically inactive, denervated muscle (1). However, expression of a small number of genes becomes altered after denervation. Some of them, including genes for acetylcholine receptor subunits, N-CAM, and myogenin, are expressed at significantly higher levels under such conditions, while the opposite was observed for some other genes like that of acetylcholinesterase (AChE; EC 3.1.1.7) (2,3,4) and α-actin (5) genes.

Decreased expression of AChE in the denervated and therefore inactive muscle is especially puzzling, since it is downregulated by both, electromechanical activity and denervation (2,6). It could be hypothesized, that electromechanical activity downregulates AChE in a specific manner and through the same mechanisms, responsible for downregulation of acetylcholine receptor subunits. On the other hand, denervation decreases AChE and its mRNA through some other events, which are obviously strong enough to override upregulating effect of electromechanical inactivity. One possible mechanism that could explain decreased level of AChE mRNA in the denervated muscle is increased rate of its degradation.

Expression of AChE in the mammalian skeletal muscle was already reported to be importantly controlled at the mRNA stability level (7). However, this report is based on the observations on the cultured mouse muscle cell line during fusion and could therefore not be simply extrapolated to the adult denervated muscle. The aim of our research is to investigate, whether decreased AChE mRNA level in the denervated muscle reflects higher capacity of AChE mRNA degradation under such conditions. Following specific questions were addressed: 1) What is the time course of AChE mRNA decrease after mechanical interruption of the motor nerve; this information is essential for understanding of the mechanisms underlying the observed AChE mRNA fall. In order to answer this question, we determined AChE mRNA levels 5 h, 18 h, 2

days, 5 days, and 8 days following denervation of rat SM muscle; 2) How is the capacity of AChE mRNA degradation distributed among muscle subcellular fractions? AChE mRNA degradation rate was determined in the postmitochondrial and polysomal fractions and in the postpolysomal supernatant of adult rat muscle; 3) What is the role of the divergent 3' untranslated region of AChE mRNA in the stabilization of mRNA against degradation in muscle subcellular compartments. Namely, AChE gene has two polyadenylation signals. Their alternate usage gives rise to two transcripts, 2.3 and 3.5 kb long. It has been reported, that additional sequence of the long transcript contains ARE elements (7). These sequences were suggested to be responsible for the control of AChE expression at the level of mRNA stability (15); 4) How specific is AChE mRNA degradation in the muscle subcellular compartments. Degradation rate of AChE mRNA was compared to the degradation rates of ribosomal RNA and of α - actin mRNA.

EXPERIMENTAL PROCEDURES

Treatment of animals and muscle preparation

Female Wistar strain albino rats, weighting about 190 g, were used. Sternomastoideus muscles (m. SM) were unilaterally denervated by the excision of a few millimeters of motor nerve close to its entry to the muscle. After 5 and 18 hours, 2, 5 and 8 days muscles were isolated. Prior the isolation rats were anesthetized with the i.p. injection of anaesthetic containing Ketanest® /Park-Davis; 100 mg/kg) and Rompun® (Bayer; 15 mg/kg). SM muscle were quickly isolated, frozen in liquid nitrogen and stored at -80 ^{o}C until used for RNA isolation. For the preparation of total RNA, used as substrate in *in vitro* degradation systems, untreated m. SM were isolated as described. Muscle subcellular fractions were prepared from fresh SM muscle.

RNA isolation and Northern blot analyses

Total RNA was isolated from the frozen SM muscles by guanidinium thiocianate procedure (8). We added two more precipitation steps and phenol extraction. Final RNA had A_{260}/A_{280} around 1.8 and A_{260}/A_{230} from 2.3-2.8.

40 μg of total RNA (from denervated muscles) or RNA recovered from degradation reactions were fractionated on the 1% denaturing formaldehyde-agarose gels, transferred to the nylon membrane (Boehringer Mannheim) and UV fixated. RNA loading was controlled by ethidium bromide staining.

Blots were prehybridized for 2-3 hours at 58°C with DIG Easy Hyb (Boehringer Mannheim), 50 μg/ml denatured herring sperm DNA and then hybridized for 18 hours at 58°C with AChE RNA probe 20-50 ng/ml (DIG Easy Hyb; 25 μg/ml denatured herring sperm DNA). The AChE RNA probe was synthesized with Riboprobe® Combination Systems (Promega) and DIG RNA Labeling Mix (Boehringer Mannheim) according to manufacturer's instructions. The synthesized RNA corresponds to nt. 370-706, part of second exon of AChE gene. Membrane was successively washed: once for 15 minutes in 2 X SSC with 0.1% SDS, 0.5 X SSC with 0.1 % SDS and 0.2 X SSC with 0.1 % SDS at room temperature and for 15 minutes at 58°C in 0.1 X SSC with 0.1 % SDS. Detection of DIG labeled probe was carried out with DIG Nucleic Acid Detection Kit, according to manufacturer's instructions, with CDP-*Star*™ as chemiluminescent substrate for alkaline phosphatase. After stripping with boiled 0.1% SDS the membrane was hybridized with human β-actin probe (Boehringer Mannheim), 10 ng of probe per ml of DIG Easy Hyb and 25 μg/ml denatured herring sperm DNA, at 58°C for 18 hours. Washing of membrane and detection of labeled probe was the same as for AChE RNA probe.

Northern blot luminographs were densitometrically analyzed by image analyzer (hardware: Imaging Research Inc., Brooke University, Ontario, Canada; software: Micro Computer Imaging Device) according to Masters et al. (9).

Preparation of muscle subcellular fractions

Fresh SM muscle was quickly homogenized (1g/ 30 ml) (20 seconds at maximal speed) with Ultra-Turrax T25 (Janke & Kunkel Labortechnik) in chilled buffer A (250 mM potasium acetate, 10 mM magnesium acetate, 2 mM dithiothreitol, 10 mM Tris-acetate, pH 7.6 (10,11,12). The homogenate was centrifuged at 15000 x *g* for 10 minutes. A part of the supernatant (postmitochondrial fraction) was quickly frozen and stored at -80°C. Centrifugation of the remaining postmitochondrial fraction at 100000 x

g for 1 hour yielded a supernatant fraction (postpolysomal fraction) and a pellet used as polysomes. Both were quickly frozen and stored at -80°C.

Analysis of RNA stability *in vitro*

Each subcellular fraction, obtained as described, was combined with total deproteinated RNA from SM muscle and incubated at 37°C. We used 60 μg of total RNA per reaction and a selected volume of subcellular fraction (see Figures). The concentrations of ions were adjusted so that the final reaction conditions were: 100 mM potasium acetate, 2 mM magnesium acetate, 2 mM dithiothreitol, 10 mM Tris-acetate, pH 7.6 (12). The degradation reactions with a certain subcellular fraction were carried out in a single tube, and 60 μl aliquots were removed at various time points. The reaction was terminated by transferring the aliquot to 500 μl of chilled guanidinium thiocianate solution. This solution was frozen and later processed as described by Chomczynsky and Sacchi (8). The RNA obtained was used for Northern blot analysis.

RESULTS AND DISCUSION

We found that AChE mRNA level remains the same during first 5h after denervation and that it abruptly falls after subsequent 13 hours. Much slower decrease rate was observed after this period of time (Fig. 1). Densitometric analyses of three independent Northern blots revealed that the longer transcript was more affected than the shorter one. The level of α-actin mRNA was also decreased in the denervated muscle and the rate of its disappearance was similar to that of AChE mRNA, suggesting that AChE mRNA is not specifically affected under such conditions. The mechanism underlying reduction in AChE mRNA level seems to be fully developed in less than one day after denervation, but needs more than 5 hours to start acting. Therefore, electromechanical activity due to the fibrillations observed occasionally in the denervated muscle could not be responsible for the observed decrease in AChE mRNA, since these fibrillations were reported to occur 3 days after denervation.

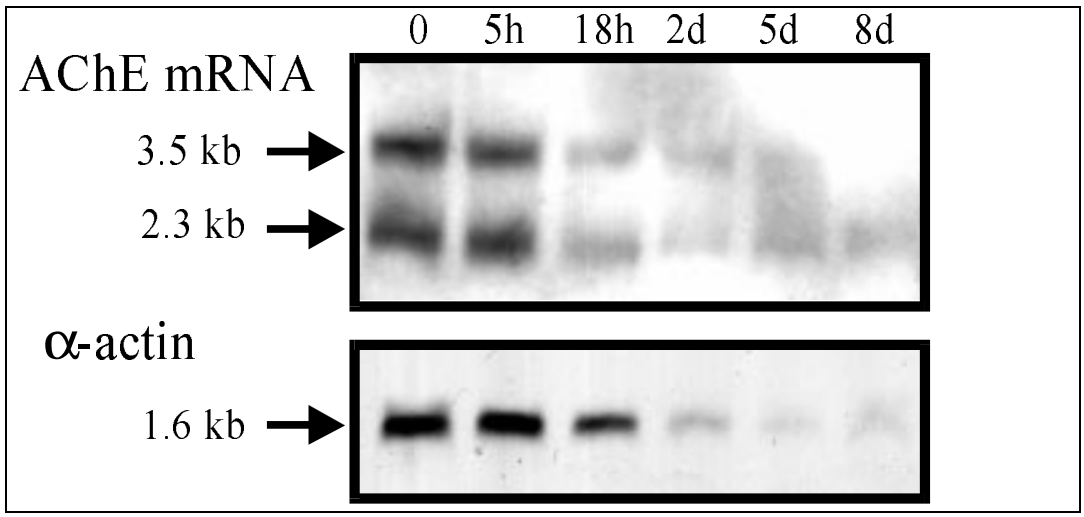

Figure 1: Northern blot analysis of AChE mRNA and α-actin mRNA from SM muscles, isolated at different times after denervation (h=hours; d=days). Equal amounts of total RNA (40 μg) were loaded (ethidium bromide staining).

Degradation of AChE mRNA was observed in *in vitro* degradation systems with all three subcellular fractions. The degradation rate was higher in postmitochondrial (Fig. 2) and postpolysomal fractions (Fig. 4) than in polysomal fraction (Fig. 3).
With a probe corresponding to nt. 370 - 706 in the second exon of AChE gene, three degradation products of approximately 1700, 1100 and 600 bases were detected (Fig. 2,3,4). Additional degradation products, not corresponding to our probe and therefore not detected, could not be excluded.

Longer transcript (3.5 kb) appeared more sensitive to degradation than the shorter one (2.3 kb). No difference in this sensitivity could be observed among subcellular fractions studied. Results from the higher degradation rate of the longer transcript leads to the conclusion that altered 3.5/2.3 ratio, observed after denervation, results from higher degradation rate of longer transcript and not its decreased transcription. Two transcripts differ in their 3'UTR, suggesting that this region, by some yet unknown mechanism mediates different stability. It has been reported that stability of many mRNA species is indeed controlled by the nucleotide sequences at their 3'UTR (13,14). In particular, the AU- rich region in this part of the molecule, demonstrated also in case of AChE mRNA (7), was found to destabilize mRNA (15).

Complete figure available in printed version

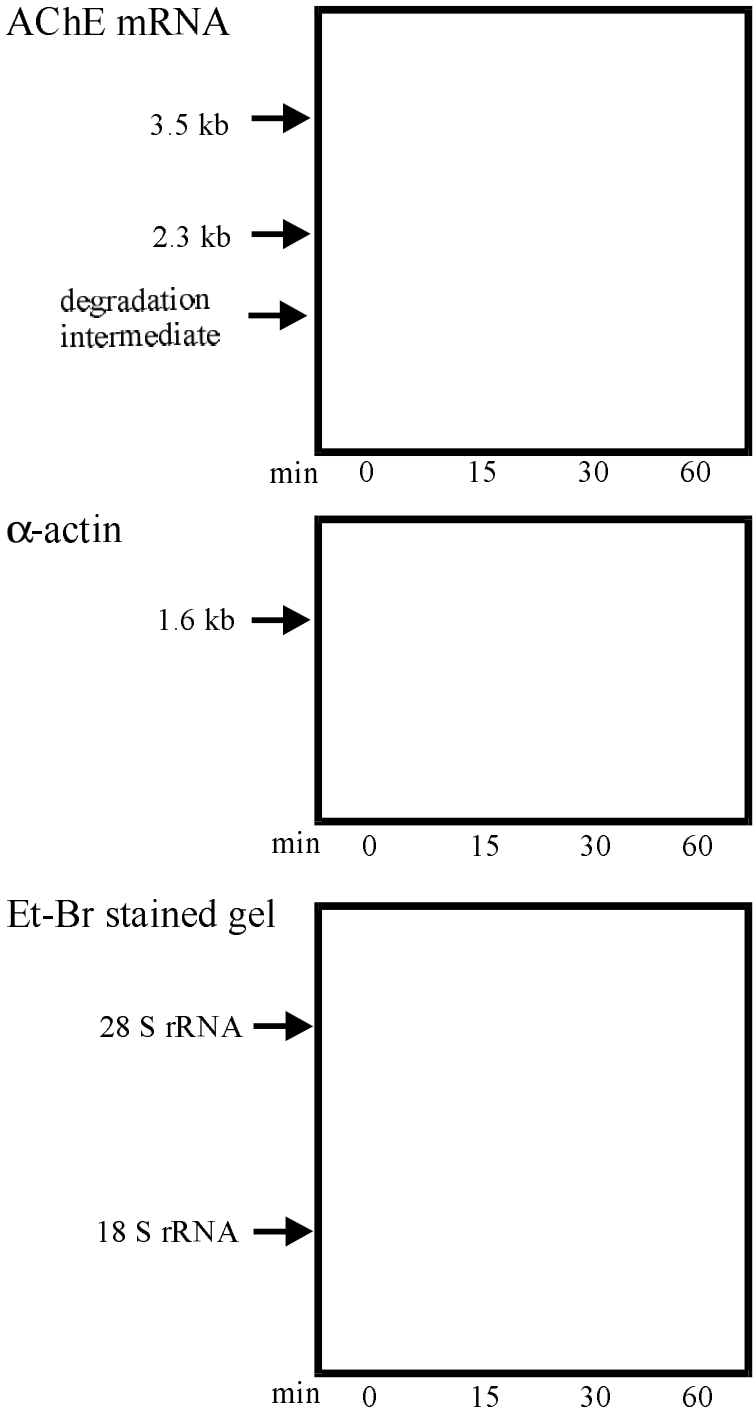

Figure 2: **Degradation with postmitochondrial fraction:** Northern blot analysis of AChE mRNA and α-actin mRNA remaining intact after selected time intervals in *in vitro* system with postmitochondrial fraction (per degradation reaction 1/200 of volume of postmitochondrial fraction obtained from one muscle). After prolonged washing following hybridization we detected 3 intermediates as in Fig.3 and 4.

Complete figure available in printed version

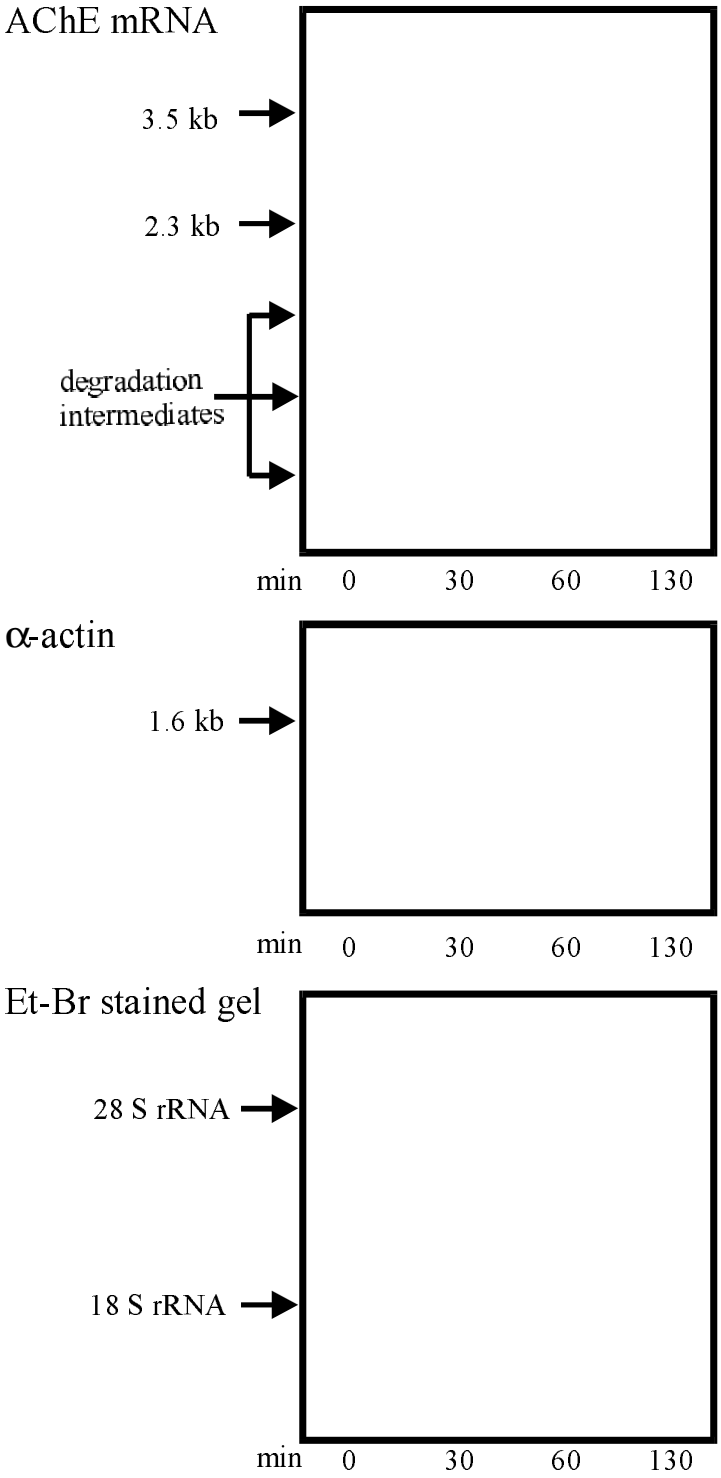

Figure 3: **Degradation with polysomal fraction:** Northern blot analysis of AChE mRNA and α-actin mRNA remaining intact after selected time intervals in *in vitro* system with polysomal fraction (per degradation reaction 1/200 of volume of polysomal fraction obtained from one SM muscle).

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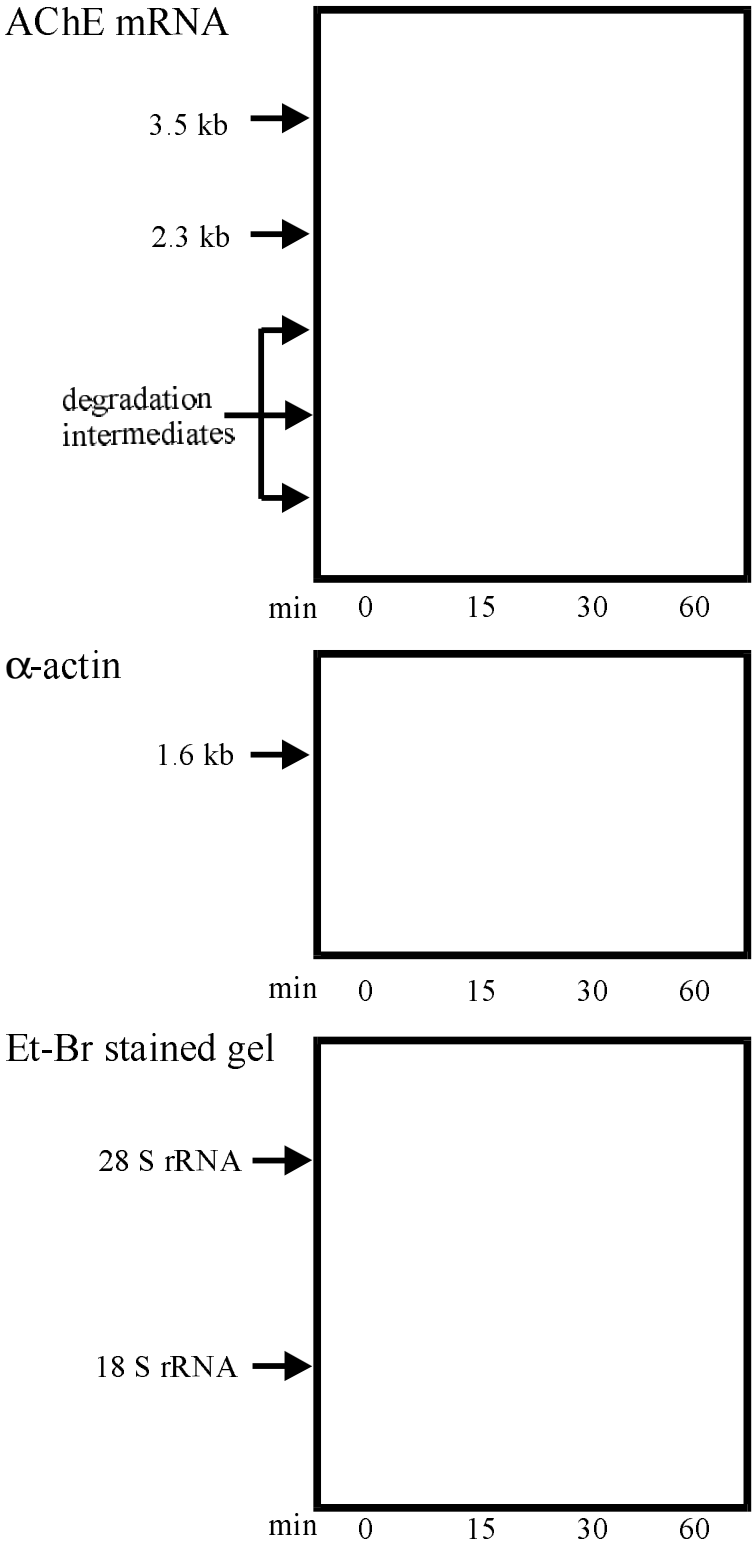

Figure 4: **Degradation with postpolysomal fraction:** Northern blot analysis of AChE mRNA and α-actin mRNA remaining intact after selected time intervals in *in vitro* system with postpolysomal fraction (per degradation reaction 1/1000 of volume of postpolysomal fraction obtained from one muscle).

The experimental approach demonstrated here, could be applied to the studies of degradation capacities of the specific mRNAs in the adult skeletal muscles. Our preliminary results suggest, that postdenervational fall of AChE transcripts is at least partly due to increased degradation susceptibility of AChE mRNA.

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POVZETEK

Tako elektromehanska aktivnost kot tudi denervacija povzročita padec ravni acetilholinesterazne (AChE) mRNA v hitri podganji skeletni mišici. Povečana hitrost razgradnje je eden od možnih mehanizmov za zmanjšanje ravni AChE mRNA v denervirani mišici. Da bi to preverili, smo iz podganje m. SM izolirali deproteinizirano RNA in jo izpostavili delovanju mišičnih subceličnih frakcij, pripravljenih iz kontralateralne m. SM. Po določenih časovnih intervalih, smo z neradioaktivno Northern blot analizo določili količino nerazgrajene AChE mRNA.

Vsebnost AChE mRNA v mišici ostane nespremenjena tekom prvih 5 ur po denervaciji, potem pa strmo pade v naslednjih 13 urah. Nadaljnje padanje je veliko počasnejše. Daljši transkript (3,5 kb) je manj stabilen kot krajši (2,3 kb).V denervirani mišici se je znižala tudi vsebnost α-aktinske mRNA, in to s podobno hitrostjo kot AChE mRNA, kar nakazuje, da AChE mRNA v takih razmerah ni prizadeta specifično. AChE mRNA se je razgrajevala z vsemi testiranimi subceličnimi frakcijami. Postmitohondrijska in postpolisomalna frakcija sta imeli večjo kapaciteto razgradnje kot polisomalna frakcija.

Ugotovili smo, da je uporabljen pristop primeren za proučevanje kapacitete razgradnje specifičnih mRNA v odrasli skeletni mišici. Naši preliminarni rezultati kažejo, da je za padec AChE mRNA po denervaciji vsaj deloma odgovorna povečana nagnjenost k razgradnji te molekule.