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Changes in the expression of mRNAs for myogenic factors and other muscle-specific proteins in experimental autoimmune myasthenia gravis

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The regulation of genes for acetylcholine receptor (AChR), myogenic factors and other muscle-specific proteins has been analyzed in experimental autoimmune myasthenia gravis (EAMG) and following denervation. The levels of the transcripts for the myogenic factors, MyoD1, myogenin and MRF4, were measured using Northern blot analysis. Myogenin and MRF4 transcript levels were observed to be 3.1- and 2.6-fold higher in muscle of rats with EAMG than in controls, respectively. MyoD1 levels, however, remained unchanged. The increases in AChR, myogenin and MRF4 mRNAs were one order of magnitude higher in 2-week denervated muscle than in the myasthenic muscle. The levels of muscle creatine kinase (MCK), α-actin and muscle dystrophin transcripts were also analyzed. Dystrophin levels were found to be 1.7- and 4.7-fold higher in EAMG and denervated muscle, respectively, than in controls; in contrast, MCK and α-actin levels remained unchanged.

Experimental autoimmune myasthenia gravis; Denervation; Myogenin, MyoD1; MRF4; Acetylcholine receptor mRNA; Muscle creatine kinase; α-Actin; Muscle dystrophin

1. INTRODUCTION

Myasthenia gravis (MG) and its animal model, experimental autoimmune myasthenia gravis (EAMG), are neuromuscular disorders characterized by weakness and fatigability of the voluntary muscle. The basic defect in MG and EAMG is a reduction of membranal acetylcholine receptor (AChR), which is the major autoantigen in this disease [1]. Regulation of AChR and its subunit mRNAs during normal muscle development is complex; the transcript levels increase during myogenic differentiation, are repressed during muscle innervation, and denervation results in their re-accumulation [2]. The changes in the levels of AChR subunit mRNAs are usually associated with increases in the transcripts of these genes, as has been observed during differentiation and after denervation of skeletal muscle [3–5]. We have previously reported that in EAMG the reduction in membranal AChR content is associated with a mild increase in AChR transcripts coding for the adult type of receptor [6,7]. We have also demonstrated an increase of AChR transcripts following a cholinergic blockage by α-bungarotoxin (α-BTX) [8]. The increases in AChR mRNA levels in EAMG and following α-BTX treatment are qualitatively smaller than those observed upon denervation.

A family of skeletal muscle-specific transcription factors that regulate the expression of a number of muscle genes during development, composed of four members known as MyoD1 [9], myogenin [10,11], myf-5 [12] and MRF4/Herculin/Myf-6 [13–15], has recently been identified. Both myogenin and MyoD1 have been reported to bind to the enhancers, and activate transcription of the AChR α- [16,17] and γ- [17,18] subunit genes, as well as the genes coding for muscle creatine kinase (MCK) [19,20], myosin light chain (MLC) [21] and troponin I [22]. Consistent with the idea that these myogenic factors may regulate AChR gene transcription in vivo, is the fact that changes in myogenin and MyoD1 levels during innervation and after denervation of skeletal muscle precede changes in AChR mRNA levels [23]. In addition, electrical stimulation of adult denervated muscle suppresses the increases of AChR, myogenin and MyoD1 mRNA levels, suggesting that contractile activity plays a role in regulating expression of myogenic factors [23]. Interestingly, the observation that blockage of neuromuscular transmission by treatment with α-BTX causes an increase in AChR and myogenin levels, while MyoD1 transcript levels remain stable [8], could suggest a differential role of neuron-derived factors in regulating AChR [24] and myogenic factor [8] expression. The mRNA expression of the other myogenic factors, MRF4 and myf-5, also increases after denervation [25,26], however, their capacity to bind and activate transcription of AChR genes has not yet been demonstrated.

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The fact that AChR transcripts were found to increase in EAMG, and the possibility that myogenic factors modulate transcription of AChR genes, prompted us to analyze myogenic factor mRNA expression in EAMG. In addition, we have also analyzed mRNA levels for three muscle-specific genes, MCK, α-actin and muscle dystrophin, all of which are developmentally regulated. Of these three proteins, MCK was shown to be trans-activated by MyoD1 and myogenin [19,20]. Muscle dystrophin is one of the dystrophin isoforms which is distributed along muscle fibers and shows a high concentration in the junctional folds [27,28]. In this study we report that the increase in AChR mRNAs in EAMG, and denervation is accompanied by an increase in myogenin, MRF4, and to a lesser extent, muscle dystrophin. In contrast, MCK mRNA and α-actin mRNA levels are not significantly different in myasthenic and denervated muscle. The increases associated with denervation are in all cases considerably higher than those in EAMG.

2. MATERIALS AND METHODS

2.1. *Animal model*

EAMG was induced in ten-week old Lewis rats by monthly injections with 40 μg of purified *Torpedo californica* AChR in complete Freund's adjuvant (CFA) as described in [6]. When myasthenic symptoms were observed, animals were sacrificed and their hindlimb muscles were immediately removed for RNA preparation. Denervation was performed as described [8] and the muscles were removed from the hindlegs one or two weeks following denervation.

2.2. *Northern blot and hybridization probes*

Total RNA was extracted as previously described [6]. Samples of RNA (30 μg each) were electrophoresed on 1% agarose formaldehyde gels and transferred to GeneScreen Plus (NEN, Du PONT) filters [6]. Hybridizations were performed using the following specific probes: the probe for AChR α-subunit was an *Eco*RI fragment derived from the mouse α-subunit recombinant DNA (kindly provided by Dr. S. Heinemann); the MyoD1 probe (kindly provided by Dr. A.B. Lassar) was a mouse cDNA fragment that was cleaved out of the vector pEMC11S with *Eco*RI; the myogenin probe (kindly provided by Dr. W.E. Wright) was a rat cDNA fragment and was cleaved out of the pESVMGN vector with *Eco*RI; the MRF4 probe was a 123 bp rat cDNA which was cleaved out of the Gemini 3 vector by *Eco*RI and *Bam*HI; the α-skeletal actin probe was a 600 bp rat cDNA; the MCK probe was an oligonucleotide (5'TGAAGCCACCATGGCGGT-CCTGGAT3'); and the dystrophin probe was a cDNA fragment of 332 bp which was subcloned into the Gemini 3 vector. Only 223 nucleotides of the resulting cRNA probe are protected by dystrophin mRNA. Autoradiograms of the Northern blots were scanned through a Molecular Dynamics 300A Computing Densitometer using the ImageQuant Software to get volume integration (total O.D. in the area of the signal). Averages of the results from 3 different blots were calculated.

2.3. *RNase protection assay*

The RNase protection assays were performed as previously described [29]. The hybridization was done at 50°C, and RNase digestion at 22°C for 75 min. The digestion products were size-fractionated on a 6% polyacrylamide/urea sequencing gel, and the gels were fluorographed at 70°C for 18–48 h.

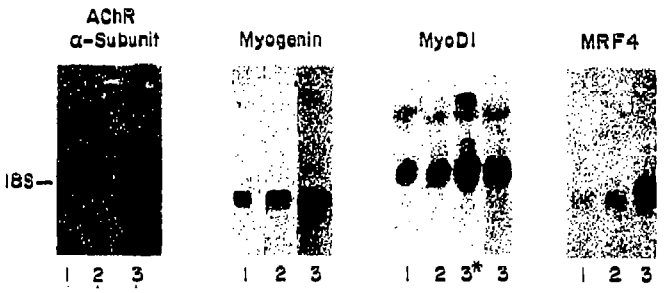

Fig. 1. Northern blot hybridization analysis of AChR α-subunit, myogenin, MyoD1 and MRF4 mRNAs in EAMG and following denervation: (1) control rat; (2) myasthenic rat; (3) denervated rat two weeks or one week following denervation. Total RNA samples (30 μg/lane) were sized-fractionated, blotted and hybridized with AChR α-subunit, myogenin, MyoD1 and MRF4 cDNA-specific probes.

3. RESULTS

We have previously demonstrated that in both EAMG- and in α-BTX-treated rats there is an increase in the levels of AChR transcripts, although membrane AChR content is reduced [6–8]. We were interested to find out whether, in addition to the increase in AChR mRNAs in EAMG, there is a change in the expression of myogenic factors. For comparison, we also measured the levels of these transcripts following denervation. Northern blots of total RNA isolated from either normal, myasthenic or denervated rat muscle were hybridized with cDNA probes coding for the myogenic factors, MyoD1, myogenin and MRF4, or for the AChR α-subunit. As shown in Fig. 1, the mRNA levels for AChR, myogenin and MRF4 are higher in EAMG and in two-week denervated muscles, than in normal muscle. MyoD1 mRNA levels, however, are approximately equal in normal, EAMG and 2-week denervated muscle. In muscle denervated for one week the MyoD1 levels are slightly higher than control. By quantitation

Table I

Expression of muscle-specific genes in EAMG and following denervation

Specific mRNA	EAMG (fold increase)	Denervation (fold increase)
AChR α-subunit	5.24 ± 1.31	56.9 ± 12.5
MCK	0.92 ± 0.17	0.81 ± 0.14
α-Actin	1.02 ± 0.35	0.76 ± 0.14
Dystrophin	1.7 ± 0.5	4.7 ± 1.4
MyoD1	1.15 ± 0.22	0.96 ± 0.14
MyoD1		1.7 ± 0.25[a]
Myogenin	3.1 ± 1.8	38.9 ± 3.6
MRF4	2.6 ± 0.7	19.3 ± 0.6

The data was obtained from quantitation of signals on Northern blots using densitometric scanning. The values have been normalized to those obtained from control muscle, and represent the fold change found in EAMG and two weeks following denervation (unless otherwise mentioned) (Mean ± S.D.). The data analyzed was obtained from three independent experiments.

[a]One-week denervation.

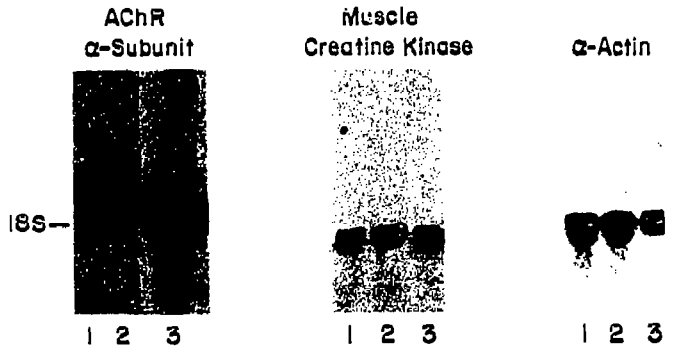

Fig. 2. Northern blot analysis of AChR α-subunit, muscle creatine kinase and α-actin mRNA in (1) control, CFA-injected rat, (2) myasthenic rat and (3) denervated rat. Total muscle RNA (30 μg/lane) was blotted and hybridized with the different specific cDNA probes.

of blots we observed in muscles from rats with EAMG a moderate increase in myogenin and MRF4 mRNA levels of 3.1- and 2.6-fold, respectively, while MyoD1 mRNA levels remained stable (Table I). As previously reported [8,23,25,30], in these experiments we also observed a large increase in the level of myogenin (39-fold) and MRF4 (19.3-fold) one week (data not shown) or two weeks following denervation (Table I). In this set of experiments MyoD1 transcripts were slightly elevated following one-week denervation, while two weeks following denervation the MyoD1 mRNA returned to its control, innervated, levels (Fig. 1, Table I). The increase of MyoD1 mRNA levels is higher shortly after denervation and drops with time, as has been shown previously in mice ([25], A.B. unpublished results). The elevation in myogenin and MRF4 is quantitatively comparable to the increase in AChR α-subunit mRNA in myasthenia, as well as upon denervation. The level of the α-subunit, myogenin and MRF4 transcripts were found to be about one order of magnitude higher in denervation than in EAMG.

The expression of myogenic factors during early stages of development [23,31] is consistent with a role of these factors in activating the expression of a large repertoire of skeletal muscle-specific genes during myogenic differentiation. These include genes coding for AChR subunits, MCK, α-actin and muscle dystrophin. In light of the fact that transcripts for two of the myogenic factors are increased in EAMG and upon denervation, we were interested to investigate if expression of other muscle specific genes is affected in EAMG and denervated muscle. Total RNA extracted from myasthenic and denervated rats was analyzed by Northern blot hybridization using oligonucleotide probes specific for MCK and cDNA probes specific for AChR α-subunit and α-actin (Fig. 2). Quantitation of blots showed that the levels of MCK mRNA, as well as α-actin, are not changed in EAMG and are slightly decreased following denervation (Table I). To analyze expression of the muscle dystrophin transcripts, RNase protection assays were performed using a cRNA probe derived from the C-terminal domain of the dystrophin cDNA that protects a region of 223 nucleotides (Fig. 3). This RNase protection analysis revealed a slight increase (1.7-fold) in EAMG and a larger increase (4.7-fold) upon denervation of the muscle dystrophin mRNA (Table I).

4. DISCUSSION

In order to understand how myogenic factors may perform different functions in vivo, it is important to analyze their temporal and local expression during development and in different biological paradigms. Previous studies have shown that MyoD1 and myogenin can regulate transcription of skeletal muscle genes which are differentially expressed during development and muscle innervation or after nerve-impulse blockade. The observations that AChR mRNA levels are higher in EAMG as compared to normal muscle, and that AChR is regulated by members of the myogenic factors, prompted us to analyze the expression of the myogenic factors in EAMG. In addition, we have compared the levels of myogenic factor mRNAs in EAMG and denervation, since partial blockage of neuro-

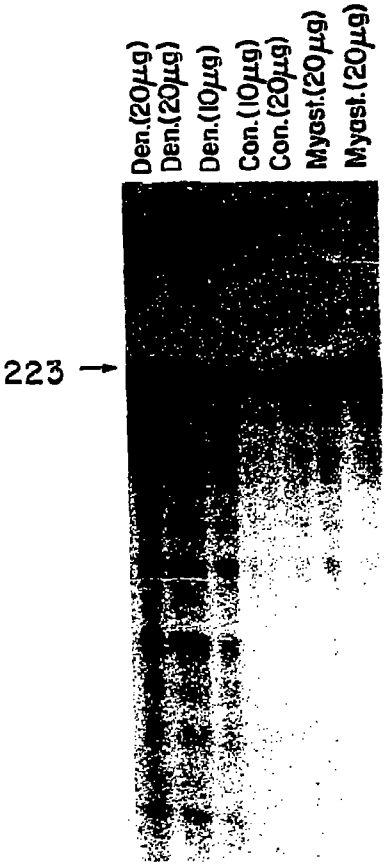

Fig. 3. RNase protection assay was performed on 20 or 10 μg RNA samples as indicated, derived from myasthenic (Myast), denervated (Den) or control (Con) muscles. The radiolabeled RNA probe used in this assay is complementary to nucleotides 9433–9656 of the rat dystrophin cDNA. A specific fragment of 223 nucleotides is protected.

transmission is observed in EAMG. In this report we have shown that MyoD1, myogenin and MRF4 levels are altered to different extents in both EAMG and upon denervation. Myogenin and MRF4 mRNA levels are higher in EAMG than in normal adult muscle, whereas MyoD1 mRNA levels are approximately the same in both conditions. The increases due to denervation, as previously shown [23,25,30], are approximately 10-fold higher. This quantitative difference between EAMG and denervation can be explained by the fact that in EAMG, as well as following application of α-BTX [8,24] or tetrodotoxin [24], the contact between the nerve and muscle is not destroyed, as opposed to denervation. Therefore, nerve-derived factors may be implicated in the regulation of myogenic factor mRNA levels in addition to muscle activity. This has been proposed for the regulation of AChR mRNAs, where total block by either denervation or botulinum toxin [24] leads to large increases in AChR transcripts. It is also possible that residual electrical activity in EAMG may be sufficient to prevent high accumulation of myogenic factor mRNAs as well as of AChR mRNAs.

In contrast to the increase of AChR α-subunit and myogenic factor mRNAs associated with EAMG and denervation, the levels of MCK transcript remained unchanged. An interesting paradox, as was previously discussed for MCK and MLC [23], is that although MyoD1 and myogenin can regulate transcription of MCK, MLC and AChR genes, MCK and MLC transcript levels do not increase after denervation. A possible explanation for these observations is that myogenin and MyoD1 may regulate the expression of MCK, MLC and AChR genes during differentiation. However, later in development in innervated muscle, MCK and MLC are regulated either by other members of the MyoD family or by other factors (i.e. MEF 2) which have been implicated in the regulation of these genes in cultured muscle cells [32]. A slight increase in the level of muscle dystrophin was observed in EAMG and a larger one following denervation. Since muscle dystrophin occurs mainly in the junctional folds [28], it might be regulated directly or indirectly by muscle activity as well as by the nerve terminal. We do not know yet whether the low increase in dystrophin message in EAMG may imply that it is also involved in the pathogenesis of myasthenia gravis. Finally, it would be interesting to know if in MG, as found in EAMG, the levels of AChR subunit mRNAs, myogenic factor and possibly muscle dystrophin mRNAs are also elevated, and if these increases are a consequence of the immune response to AChR.

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