Preclinical increase in activity of muscle microsomal trypsin-like protease in murine muscular dystrophy, C57BL/10-mdx

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Preclinical alterations of protease activities in skeletal muscles from 10-day-old dystrophic mouse, C57BL/10-mdx, were examined by using 10 fluorogenic peptide substrates. Among the activities tested, only Boc-Val-Pro-Arg-MCA-hydrolyzing enzyme of the muscle microsomes showed an about 6-fold higher level of activity in mdx mouse. The increase in activity was not observed in tissues other than skeletal muscle. The enzyme had a pH optimum between 8.5 and 11.0, and was inhibited with DFP and variety of trypsin inhibitors. The enzymatic activity transiently increased at 1–2 weeks of age, the preclinical or very early stage of the disease. These results imply that the increased level of a trypsin-like protease possibly present in muscle microsomes may be closely related to the manifestation of muscular dystrophy.

Muscular dystrophy *Protease* *Trypsin-like enzyme* *Enzyme activity* *(mdx mouse)*

1. INTRODUCTION

A primary cause of muscular dystrophy has not yet been elucidated. To demonstrate the cause of the disease, it is indispensable to study the biochemical changes not in the affected muscles, but in muscles just before the onset of the disease, because a variety of secondary lesions would be elicited by the primary lesion. It is well known that some muscle proteases participate in the progress of muscular dystrophy [1–3]. However, little or no information is available on the preclinical alteration of muscle protease activity.

C57BL/10-mdx is a recently discovered murine muscular dystrophy [4]. The animal is an X-linked recessive mutant, bearing close resemblance to human Duchenne muscular dystrophy on the basis of gene locus on each X chromosome [4]. Since the animal is fertile, preclinical mice, in which histological lesion certainly begins to occur in muscles at 15–20 days of age [4–6], are easily obtained by matings of mdx homozygotes and hemizygotes. The feature enabled us to examine the preclinical alterations of muscle protease activities of the mdx mouse.

Here, we present evidence for a remarkable increase in activity of Boc-Val-Pro-Arg-MCA-hydrolyzing enzyme in muscle microsomes of mdx mouse at the age just before the onset of histological lesion in muscles.

2. MATERIALS AND METHODS

2.1. *Animals*

The murine muscular dystrophy, C57BL/10-mdx, was supplied by Dr K. Esaki of Central Institute for Experimental Animals. Normal mice, C57BL/10 were obtained from Shizuoka Laboratory Animal Center.

2.2. *Chemicals*

Peptidyl-4-methylcoumaryl-7-amide (MCA), 7-amino-4-methylcoumarin (AMC), antipain, chymostatin, elastatinal and E-64 were purchased

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from the Peptide Institute. Leupeptin and bestatin were gifts from Dr T. Aoyagi of the Institute of Microbial Chemistry. The other protease inhibitors were from Sigma.

2.3. *Enzyme preparation*

Animals were killed by decapitation. Hind-limb muscles were immediately excised, washed and homogenized in 10 vols of 0.32 M sucrose (pH 7.4)

Table 1

Protease activities of mitochondrial and lysosomal, microsomal, and cytosol fractions of hind-limb muscles from 10-day-old normal and mdx mice

Substrate P5 P4 P3 P2 P1	Subcellular fraction	Activity		(B)
		Normal (A)	mdx (B)	(A)
Boc-Val-Pro-Arg-MCA	mit. & lys	0.081 ± 0.014	0.171 ± 0.055	2.1[a]
	microsomes	0.030 ± 0.007	0.172 ± 0.030	5.7[b]
	cytosol	0.341 ± 0.052	0.378 ± 0.079	1.1
Boc-Phe-Ser-Arg-MCA	mit. & lys.	0.629 ± 0.154	0.477 ± 0.086	0.76
	microsomes	0.059 ± 0.004	0.063 ± 0.025	1.1
	cytosol	0.043 ± 0.002	0.054 ± 0.007	1.3[a]
Z-Phe-Arg-MCA	mit. & lys.	4.23 ± 0.39	5.09 ± 0.88	1.2
	microsomes	0.340 ± 0.007	0.290 ± 0.023	0.85
	cytosol	0.221 ± 0.031	0.285 ± 0.038	1.3
Z-Arg-Arg-MCA	mit. & lys.	0.595 ± 0.175	0.533 ± 0.070	0.90
	microsomes	0.051 ± 0.005	0.055 ± 0.009	1.1
	cytosol	0.046 ± 0.004	0.051 ± 0.006	1.1
Suc-Leu-Leu-Val-Tyr-MCA	mit. & lys.	0.059 ± 0.010	0.071 ± 0.006	1.2
	microsomes	0.140 ± 0.026	0.105 ± 0.038	0.75
	cytosol	0.046 ± 0.004	0.062 ± 0.016	1.3
Suc-Ala-Ala-Pro-Phe-MCA	mit. & lys.	0.010 ± 0.001	0.013 ± 0.003	1.3
	microsomes	0.010 ± 0.001	0.015 ± 0.006	1.5
	cytosol	0.184 ± 0.055	0.194 ± 0.041	1.1
Suc-Ala-Pro-Ala-MCA	mit. & lys.	0.012 ± 0.004	0.009 ± 0.007	0.75
	microsomes	0.011 ± 0.003	0.016 ± 0.005	1.5
	cytosol	0.017 ± 0.003	0.021 ± 0.004	1.2
Gly-Pro-MCA	mit. & lys.	0.658 ± 0.095	0.988 ± 0.163	1.5[c]
	microsomes	0.956 ± 0.057	0.875 ± 0.147	0.92
	cytosol	0.273 ± 0.043	0.324 ± 0.059	1.2
Leu-MCA	mit. & lys.	0.850 ± 0.232	1.37 ± 0.61	1.6
	microsomes	1.43 ± 0.18	1.31 ± 0.39	0.92
	cytosol	2.59 ± 0.13	2.56 ± 0.51	0.99
Arg-MCA	mit. & lys.	0.998 ± 0.144	1.13 ± 0.30	1.1
	microsomes	1.26 ± 0.20	1.53 ± 0.19	1.2
	cytosol	2.79 ± 0.83	2.77 ± 0.41	0.99

Enzymatic activities are expressed as mean ± SE nmol AMC/min per mg protein. Degree of significance is as follows: [a]$P<0.05$, [b]$P<0.002$, [c]$P<0.02$. mit. & lys., mitochondria and lysosomes; Boc, *t*-butyloxycarbonyl; MCA, 4-methylcoumaryl-7-amide; Z, benzyloxycarbonyl; Suc, succinyl

with a Teflon homogenizer in an ice bath. After the post-nuclear fraction (700 × *g*, 10 min) had been centrifuged at 14000 × *g* for 30 min, the supernatant was recentrifuged at 100000 × *g* for 90 min. Subcellular fractions of 14000 × *g* precipitate suspended in acetate buffer (pH 5.0), 100000 × *g* precipitate suspended in Tris buffer (pH 7.4) and 100000 × *g* supernatant were taken as the fractions of mitochondria and lysosomes, microsomes, and cytosol, respectively, without further purification.

2.4. *Enzyme assay*

The enzymatic activities were determined fluorophotometrically using peptidyl-MCA as substrates [7]. The reaction mixture (0.5 ml) contains 10 μM peptidyl-MCA, 10 mM 2-mercaptoethanol, 50 mM Tris-HCl (pH 7.4), and a portion of enzyme solution (20–50 μl). After incubation at 25°C for 30–60 min, the reaction was terminated by the addition of 2 ml of 0.1 M sodium acetate (pH 4.3) containing 0.1 M sodium monochloroacetate [8].

2.5. *Measurement of protein concentration*

Protein concentration was determined by the method of Lowry et al. [9] with bovine serum albumin as a standard.

Table 2

Boc-Val-Pro-Arg-MCA-hydrolyzing enzyme activities of microsomal fractions from various tissues of normal and mdx mice

Tissue	Activity		(B)
	Normal (A)	mdx (B)	(A)
Skeletal muscle	0.025	0.197	7.9
Heart	0.036	0.033	0.92
Lung	0.233	0.153	0.66
Liver	0.020	0.016	0.80
Kidney	0.029	0.031	1.1
Brain	0.010	0.011	1.1
Spleen	0.009	0.011	1.2

Each tissue from 10-day-old normal and mdx mice (3–4 individuals) was rinsed with physiological saline, homogenized and fractionated as described in the text. Enzymatic activities are expressed as nmol AMC/min per mg protein

3. RESULTS

3.1. *Differences of protease activities between normal and mdx mice at 10 days of age*

Ten peptidyl-MCA-hydrolyzing enzyme activities of the three subcellular fractions of hind-limb muscle from 10-day-old normal ($n=5$) and mdx ($n=4$) mice were compared with each other in table 1. As shown in the table, only Boc-Val-Pro-Arg-MCA-hydrolyzing enzyme of the microsomal fractions showed a markedly (6-fold) higher level of activity in mdx mouse, while the other activities showed little or no significant differences between

Table 3

Effects of reagents on muscle microsomal Boc-Val-Pro-Arg-MCA-hydrolyzing enzyme activity from 10-day-old mdx mouse

Reagent	Concentration	% activity
None	–	100
DFP	1 mM	0
PMSF	1 mM	27
EDTA	1 mM	89
PCMB	1 mM	108
E-64	10 μM	97
Leupeptin	10 μM	48
Antipain	10 μM	5
Chymostatin	10 μM	100
Elastatinal	10 μM	100
Bestatin	10 μM	101
STI	1 mg/ml	115
LBTI	1 mg/ml	129
Antithrombin III	1 unit/ml	59
Hirudin	1 unit/ml	4
Ovomucoid	1 mg/ml	59
Aprotinin	1 mg/ml	49
α_1-Antitrypsin	1 mg/ml	60
$CaCl_2$	1 mM	104
$MgCl_2$	1 mM	104
2-Mercaptoethanol	10 mM	99
Brij 35	0.01%	119

The enzyme was preincubated with each reagent at an indicated concentration for 30 min at 25°C, pH 7.4. Residual activity of the enzyme was determined by adding small amount of the substrate solution at a final concentration of 10 μM into the reaction mixture. Abbreviations: DFP, diisopropyl fluorophosphate; PMSF, phenylmethanesulfonyl fluoride; PCMB, *p*-chloromercuribenzoic acid; STI, soybean trypsin inhibitor; LBTI, lima bean trypsin inhibitor

them. The marked alteration in Boc-Val-Pro-Arg-MCA-hydrolyzing activity of the microsomal fraction was not observed in tissues other than skeletal muscle (table 2).

3.2. *Enzymatic properties*

The optimum pH of the muscle enzyme from 10-day-old mdx mouse was between 8.5 and 11.0. The enzyme was inhibited with DFP, PMSF and a variety of trypsin or thrombin inhibitors except STI and LBTI, but not or scarcely affected by PCMB, EDTA, chymostatin, elastatinal, bestatin, $CaCl_2$, $MgCl_2$, 2-mercaptoethanol and Brij 35 (table 3). Thus, the enzyme is a trypsin-like or thrombin-like serine protease, and is active under the physiological pH and Ca^{2+} concentration. An apparent K_m of the enzyme toward Boc-Val-Pro-Arg-MCA was determined to be 8.1 μM at pH 7.4.

3.3. *Boc-Val-Pro-Arg-MCA-hydrolyzing enzyme activities in muscle microsomes from normal and mdx mice at different ages of the preclinical state*

Activities of muscle microsomal Boc-Val-Pro-Arg-MCA-hydrolyzing enzyme from normal and mdx mice were determined at different ages between 2 and 15 days (fig.1). The enzymatic activity in mdx mouse began to significantly increase at 1 week of age and reached a maximum value at 10 days of age, and then reduced. These results indicate that the activity is temporally increased at the age just before the onset of histological lesion in muscles [4–6]. Thus, we consider that a transient elevation of muscle microsomal trypsin-like protease activity may be closely associated with the occurrence of murine muscular dystrophy.

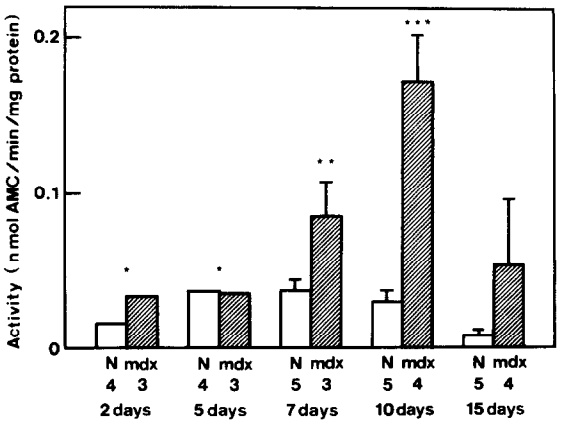

Fig.1. Boc-Val-Pro-Arg-MCA-hydrolyzing enzyme activites in muscle microsomes from normal and mdx mice at different ages between 2 and 15 days. The numbers indicated at the bottom of the columns represent the number of animals tested. Open and shaded columns indicate normal and mdx mice, respectively. *Activity of the mixture of the enzyme preparation obtained from 3–4 individuals. $^{**}P < 0.05$, $^{***}P < 0.002$.

4. DISCUSSION

We have demonstrated for the first time that the activity of a muscle microsomal protease, which hydrolyzes Boc-Val-Pro-Arg-MCA, remarkably increases in mdx mouse at the preclinical stage. Boc-Val-Pro-Arg-MCA is known as a specific substrate toward thrombin [10] and the sperm trypsin-like enzyme, spermosin [7]. The muscle protease found here is however, distinct from these two enzymes in susceptibilities toward inhibitors such as leupeptin [11] and soybean trypsin inhibitor [7]. Our enzyme is also distinct from a neutral protease of microsomal fractions of rat skeletal muscle [12] on the basis of optimum pH and inhibitor spectrum, suggesting the possibility of a novel type of protease. Alternatively, it also seems possible that the microsomal trypsin-like activity is due to tryptase of mast cells present in connective tissues of muscles, since the inhibitor spectrum of the former enzyme is essentially similar to that of the latter enzyme [13]. Purification and further characterization, as well as examination of localization, of the enzyme will solve this problem.

Muscle microsomal Boc-Val-Pro-Arg-MCA-hydrolyzing activity elevated at the age just before the appearance of histological lesion in muscles [4–6]. These results prompt us to consider the possible participation of the enzyme in the onset of muscular dystrophy. The idea is not at variance with the result of no alteration in the enzymatic activity in cardiac muscle (see table 2), since histological lesion occurs in skeletal muscle but not in cardiac muscle of mdx mouse by the 4th month of age [6].

It is well known that the activity of cathepsin B and L, probably derived from invading cells such as macrophages, increases in muscular tissues from the early stage of muscular dystrophy [1,2,14]. We also confirmed this phenomenon in mdx mouse

after 20 days of age (unpublished), indicating that the activity increase of our enzyme precedes the occurrence of elevation of cathepsin B and L activity. In addition, our preliminary experiment showed that there is also a preclinical alteration in the microsomal trypsin-like activity in one-third or a half of mdx heterozygotes. Both of these results further support the above assumption that the microsomal protease may be involved in the early event or primary cause, rather than the secondary consequences, of muscular dystrophy, though the present data have not yet been documented convincingly.

It has been reported that the administration of leupeptin to the dystrophic mouse, C57BL/6J (dy^{2J}/dy^{2J}), before the onset of muscle weakness inhibits the appearance of clinical signs of weakness as well as those of histological lesion [15]. These findings may imply the involvement of a putative trypsin-like protease in the manifestation of muscular dystrophy.

Further studies on the effects of trypsin inhibitors on the onset of muscular dystrophy in mdx mouse will clearly demonstrate the patho-physiological role of the enzyme.

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