

Release of Intracellular Enzymes from Skeletal Muscles and Diaphragm in Mdx Mice

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Abstract

Male mutant C57BL/10 mdx mice and control normal mice of the C57BL/6J strain at different ages were used to study creatine kinase (CK) and lactate dehydrogenase (LDH) release in vitro from hind limb and diaphragm muscles. The muscle specimens were incubated in Krebs-Ringer bicarbonate solution without any supplementation and in the presence of different agents which are able to change muscle cell's calcium homeostasis.

The data provide an evidence for an increased muscle plasmalemma permeability, both of mdx skeletal muscles as well as mdx diaphragm in adult animals. In young animals the release was even lower than in controls. High K⁺ concentration and calcium ionophore A23187 were affecting the plasmalemma permeability, especially in the mdx diaphragm, to a lesser degree but also evidently in the mdx skeletal muscles. Dantrolene and Verapamil were able to act on the enzymes release both from normal skeletal muscle and diaphragm, as well as from mdx muscle specimens. In the latter their action was, however, less evident as compared to normals. Their action was decreasing with age.

These results may indicate a role of intracellular calcium accumulation and its distribution on the degree of plasmalemma permeability of mdx muscles. The reaction to calcium blockers has a tendency to decrease as the disease is progressing. To prevent further impoverishment of the dystrophic muscle in some of its components, they should be applied as early as possible.

Key words: mdx mice, enzyme release, calcium homeostasis.

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Duchenne and Becker muscular dystrophies are progressive muscle wasting diseases for which the defective gene has been identified [28] and the gene product called "dystrophin" has been characterized [18]. This protein is absent or greatly diminished in Duchenne dystrophy and mdx mouse skeletal and diaphragm muscles; greatly diminished dystrophin concentration often with a changed molecular weight in Becker dystrophy is present. It is proposed that this protein exerts either a stabilizing effect on the muscle membrane [22] and/or is involved in calcium [19] and ion channels regulations [8, 11]. It is also known that calcium augments enzyme leakage from skeletal muscles [34, 35]. There is currently no known efficient therapy of these diseases. The membrane and Ca²⁺-related theories led to therapeutic trials with Ca²⁺-channel blockers as Nifedipine [5, 29], Diltiazem [2, 3, 30] and Verapamil [9, 41]. In Duchenne dystrophy they resulted in a mild improvement in cardiac parameters [2], lowering of

intracellular skeletal muscle calcium accumulation [30], short-term benefit in muscle strength and lowering the intracellular enzymes release. In mdx mice Dantrolene administration is reported to cause reductions of serum creatine kinase [32].

The beneficial effect of agents acting on muscle cell calcium concentration and its distribution inside the muscle cell seems to be of limited value, yet. It is possible, however, that it is the consequence that they are administered too late, when already evident clinical symptoms are present. In this study we examined the influence of different agents able to handle the intracellular calcium content (in positive and negative means), on the release of two muscle enzymes of different molecular weights (creatine kinase and lactate dehydrogenase) from two differently affected muscles (skeletal muscles and diaphragm) before and after muscle cell degeneration and regeneration processes are taking place.

Materials and Methods

Male mutant C57BL/10 mdx mice, 14 days to 12 months of age, and control age-matched mice of the C57BL/6J strain, were used. Four animals in each group were fed a standard laboratory diet, they were killed by cervical dislocation in ether anaesthesia and gastrocnemius and diaphragm muscles were carefully removed. The muscles were mounted at approximately resting length on a plastic support of special holders and placed in Krebs-Ringer bicarbonate buffer (pH 7.4) supplemented with 10 mM glucose and 0.5 mM cycloheximide [20]. Addition of different agents were made prior to placing the muscles samples in tubes. To the Krebs-Ringer bicarbonate buffer the following was added: 1) 100 mM KCl instead of the 100 mM NaCl, 2) Calcium ionophore A23187, 10 µg per ml, 3) Dantrolene, 10 µg per ml and, 4) Verapamil 200 µM. The tubes were placed in a shaking water bath for 30 min intervals at 37°C. At 30 min intervals the medium in each tube was removed and replaced with fresh medium warmed before to 37°C. Incubation was allowed to proceed for two hours. Bovine serum albumin (0.1%) was added to each medium in order to stabilize the enzymes activity and minimize their losses.

Creatine kinase (CK) activity was estimated by an enzyme linked assay, the reduction of NADPH being followed at 340 nm (Cormay Diagnostic Kit).

Lactate dehydrogenase (LDH) activity was measured in the direction of pyruvate to lactate formation by following the oxidation of NADH at 340 nm (Cormay Diagnostic Kit). All analyses were carried out at 25°C and the activities were expressed as IU per mg of muscle protein incubated.

The total protein content in the particular muscle samples, before the incubation was taking place, was determined by the method of Lowry et al. [23], as modified by Peterson [31].

The statistical significance of the results was assessed using Student's test and Duncan's multiple range test (GLM). A P value > 0.05 being considered as non-significant.

Results

The CK and LDH release from normal hind limb muscles and diaphragm in mdx mice and controls is presented (Table 1). In control mice no age-dependence of the efflux was observed, in this group a mean of all determinations is given. The CK release from normal skeletal muscles was always lower than from the normal diaphragm. The opposite was observed for LDH in normal skeletal and diaphragm muscles. During the first 30 minutes of incubation about 30% of the total release was present, both in normals as well as mdx animals. A total release or changes of the release as compared to those in not supplemented medium during the two hours of incubation is presented in the Tables.

The enzymes release from mdx muscles of young mdx animals was lower than in controls, in adult skeletal

muscles it was increasing progressively, achieving the top between 90 to 180 days of life of the animals. In mdx diaphragm after initially lower release in young animals, the highest efflux was also present at the age between 90 and 180 days of life, afterwards it returned, however, to that of normals. An additional and significant enzymes release from control skeletal and diaphragm muscles was induced in the presence of high K⁺ concentration (Table 2). In these conditions the CK efflux from normal diaphragm was higher than that from normal skeletal muscles, the opposite was observed for LDH. In skeletal muscles and diaphragm of young mdx animals the enzymes release in the presence of high K⁺ was lower than normally. In adult mdx mice the additional CK release in the presence of high K⁺ was significantly higher than in normals. The changes of the LDH release from mdx muscles as compared to normals were less evident. Calcium ionophore induced a significant increase of the enzymes efflux from normal skeletal muscles and normal diaphragm (Table 3). The CK efflux from normal skeletal muscle was in this conditions comparable to that from diaphragm, that of LDH was higher from skeletal muscles than from the diaphragm. No age-dependence of the release in normal animals was observed. In the mdx skeletal muscles and mdx diaphragm a significantly higher CK efflux was present both in young, as well as adult mdx animals, the top of the increase was observed between 90 and 180 days of life. The LDH efflux in the presence of calcium ionophore was less increased, as compared to that of CK. Dantrolene when added to the incubation medium lowered the enzymes release both from normal skeletal muscles, as well as from normal diaphragm (Table 4). No age-dependence of the enzymes efflux decrease was observed. The decrease of CK efflux was significantly higher when normal skeletal muscles were tested, as compared to the normal diaphragm. The degree of LDH efflux decrease in the presence of Dantrolene was similar in normal skeletal muscles and normal diaphragm. In mdx muscles Dantrolene inhibited significantly the CK release when compared to that in the not-supplemented medium, the action was, however, significantly lower, especially in adult mdx animals. The decrease in the reaction to Dantrolene was progressing with age. The decrease of LDH efflux in the presence of Dantrolene was similar in mdx skeletal and diaphragm muscles to that in normals, except of the mdx animals at the age at 180 and 360 days where the reaction to Dantrolene was already smaller. Verapamil decreased significantly the CK and LDH efflux from normal skeletal muscles and diaphragm in a similar manner (Table 5). The change in the enzymes release in the presence of Verapamil in normals was not age-dependent. The decrease in the CK efflux in the presence of Verapamil in mdx animals was significant when compared to their efflux without Verapamil supplementation, it was significantly less expressed, however, especially in the mdx diaphragm when compared to normals. The reaction of enzymes release to Verapamil was decreasing with age of the mdx animals. Verapamil

Muscle plasmalemma permeability in mdx mice

Table 1. Creatine kinase and lactate dehydrogenase release from skeletal muscles and diaphragm in vitro.

	Age days	Creatine kinase		Lactate Dehydrogenase	
		Skeletal muscles	Diaphragm IU per mg muscle proteins incubated	Skeletal muscles	Diaphragm
Mdx mice	14	260.3 ± 158.5	209.3 ± 107.4*	23.9 ± 9.8*	18.5 ± 3.6*
	30	494.4 ± 122.5	498.7 ± 128.6*	32.5 ± 8.1*	17.2 ± 0.7*
	90	1074.1 ± 116.2*	725.5 ± 116.8*	137.5 ± 21.2*	126.5 ± 38.3*
	180	939.4 ± 67.7*	829.7 ± 116.4*	399.4 ± 62.8*	120.5 ± 11.5*
	240	854.2 ± 65.6*	567.5 ± 110.9*	190.7 ± 32.5*	42.9 ± 7.8*
	360	666.1 ± 54.9*	460.9 ± 115.5*	199.6 ± 22.5*	46.2 ± 14.5*
Control mice	14 - 360	349.6 ± 97.7	553.2 ± 76.4	93.2 ± 8.1	54.9 ± 5.7

Values are means ± SD.

* Significant at P < 0.05 as compared to controls.

Table 2. Increase of creatine kinase and lactate dehydrogenase release from skeletal muscles and diaphragm in vitro in the presence of high K⁺ concentration.

	Age days	Creatine kinase		Lactate Dehydrogenase	
		Skeletal muscles	Diaphragm IU per mg muscle proteins incubated	Skeletal muscles	Diaphragm
Mdx mice	14	316.7 ± 67.7	165.6 ± 17.8*	29.0 ± 2.9*	16.3 ± 2.8
	30	837.1 ± 349.9*	1423.6 ± 485.1*	91.9 ± 17.6	28.8 ± 8.1
	90	884.7 ± 74.4*	1546.0 ± 391.9*	80.3 ± 5.5	93.5 ± 11.4*
	180	876.9 ± 389.0*	1848.5 ± 305.9*	189.5 ± 23.5*	37.4 ± 6.8*
	240	870.7 ± 197.7*	2131.6 ± 721.4*	224.0 ± 39.2*	31.4 ± 9.4*
	360	818.2 ± 264.7*	1310.3 ± 105.6*	244.9 ± 55.6*	30.5 ± 2.9*
Control mice	14 - 360	565.5 ± 112.4	1125.2 ± 342.3	73.4 ± 10.5	20.0 ± 2.6

Values are means ± SD.

* Significant at P < 0.05 as compared to the increase of the enzymes release in controls in the presence of high K⁺ concentration.

Table 3. Increase of creatine kinase and lactate dehydrogenase release from skeletal muscles and diaphragm in vitro in the presence of calcium ionophore A23187.

	Age days	Creatine kinase		Lactate Dehydrogenase	
		Skeletal muscles	Diaphragm IU per mg muscle proteins incubated	Skeletal muscles	Diaphragm
Mdx mice	14	90.1 ± 4.8*	143.1 ± 13.6*	12.9 ± 3.4	2.2 ± 0.4
	30	124.6 ± 21.1*	1478.4 ± 314.2*	24.4 ± 7.2	5.5 ± 1.3
	90	211.0 ± 88.0*	1589.1 ± 217.8*	72.1 ± 13.6*	53.7 ± 17.8*
	180	245.8 ± 88.5*	1303.7 ± 398.2*	66.4 ± 8.8*	10.9 ± 4.1*
	240	134.2 ± 67.5*	1072.9 ± 835.1*	67.7 ± 16.2*	2.9 ± 1.8
	360	132.0 ± 6.2*	142.8 ± 40.9*	71.9 ± 8.5*	1.9 ± 0.6
Control mice	14 - 360	53.9 ± 4.5	31.6 ± 4.7	15.0 ± 2.3	2.8 ± 1.7

Values are means ± SD.

* Significant at P < 0.05 as compared to the increase of the enzymes release in controls in the presence of calcium ionophore A23187.

Muscle plasmalemma permeability in mdx mice

Table 4. Decrease of creatine kinase and lactate dehydrogenase release from skeletal muscles and diaphragm in vitro in the presence of Dantrolene.

	Age days	Creatine kinase		Lactate Dehydrogenase	
		Skeletal muscles	Diaphragm IU per mg muscle proteins incubated	Skeletal muscles	Diaphragm
Mdx mice	14	121.8 ± 18.3*	18.3 ± 0.7	21.1 ± 6.8	18.1 ± 1.2
	30	131.9 ± 18.6*	9.1 ± 3.5*	31.5 ± 6.3	18.1 ± 0.8
	90	142.4 ± 11.8*	10.8 ± 4.8*	23.5 ± 3.0	16.4 ± 2.8
	180	54.9 ± 15.2*	10.3 ± 6.1*	13.5 ± 4.0*	8.2 ± 0.6*
	240	45.6 ± 16.9*	1.8 ± 0.6*	11.9 ± 2.9*	8.1 ± 1.1*
	360	35.3 ± 4.6*	1.4 ± 0.3*	7.1 ± 1.9*	11.4 ± 2.1*
Control mice	14 - 360	178.1 ± 9.1	26.5 ± 3.7	22.2 ± 3.7	28.1 ± 6.9

Values are means ± SD.

* Significant at $P < 0.05$ as compared to the decrease of the enzymes release in controls in the presence of Dantrolene.

Table 5. Decrease of creatine kinase and lactate dehydrogenase release from skeletal muscles and diaphragm in vitro in the presence of Verapamil.

	Age days	Creatine kinase		Lactate Dehydrogenase	
		Skeletal muscles	Diaphragm IU per mg muscle proteins incubated	Skeletal muscles	Diaphragm
Mdx mice	14	58.9 ± 13.4*	24.2 ± 8.1*	14.6 ± 2.2*	10.5 ± 3.7
	30	69.3 ± 26.3*	20.9 ± 12.5*	13.9 ± 2.5*	14.3 ± 1.9
	90	57.8 ± 17.9*	12.3 ± 2.0*	13.1 ± 0.1*	14.5 ± 4.2
	180	55.9 ± 7.8*	12.4 ± 3.8*	11.3 ± 2.7*	16.8 ± 3.1
	240	35.1 ± 7.4*	7.5 ± 4.3*	3.3 ± 2.2*	20.1 ± 5.4
	360	29.0 ± 8.2*	14.0 ± 3.7*	2.4 ± 1.3*	12.6 ± 5.6
Control mice	14 - 360	123.1 ± 16.3	133.7 ± 8.8	24.2 ± 5.4	14.9 ± 3.6

Values are means ± SD.

* Significant at $P < 0.05$ as compared to the decrease of the enzymes release in controls in the presence of Verapamil.

was also acting on the LDH efflux from mdx skeletal muscles although less evident as compared to normals, in the mdx diaphragm the LDH efflux in the presence of Verapamil was comparable to that of normals.

Discussion

In muscle pathology, especially in humans muscular dystrophy, a substantial release of intracellular enzymes, notably of creatine kinase, from dystrophic muscle is a well known phenomenon. The CK activity is also increased in serum of mdx mice [14, 27, 32]. According to some [27] CK hyperenzymemia is present only in adult animals. It is also reported that mdx muscles were found to release reduced amounts of CK as compared to controls in response to excessive contractile activity [25]. These latter two reports contradict the suggestion on the existence of increased plasmalemma permeability to CK from the birth of the mdx animals, at least, and also on the presence of increased susceptibility of the plasmalemma to stresses of contraction [25]. The latter report contradicts also the

suggestion that the changes in membrane permeability occur at about the age when muscle activity increases. Another characteristic feature of the Duchenne dystrophic process is the calcium accumulation in the muscle cell. Both in Duchenne dystrophy, as well as mdx mice, an elevated muscle calcium content proceed degradation of muscle proteins [4, 10, 14, 27, 38, 39]. The elevation of free intracellular calcium is proposed to be caused by increased activity of calcium leak channels in the dystrophin-deficient plasmalemma [39] and increased plasmalemma permeability, restricted however only to adult mdx animals [27]. It is also reported that in mdx mice Ca^{2+} is leaking from the sarcoplasmic reticulum, and this would accelerate further muscle degeneration [36]. Changes in intracellular calcium concentration may be also connected with the content of dystrophin. There are evidence provided for the involvement of dystrophin in stabilization of calcium-sensitive stretch inactivated ion channels [11]. There are, however, some uncertainties concerning the role

of intracellular calcium in the pathologic processes in dystrophy. It is demonstrated that no increased influx of ^{45}Ca into intact dystrophin-deficient muscles in vitro is present [26], there is a comparable CK release in the presence of calcium ionophore in mdx mice and controls [25], and also normal levels of intracellular free calcium in intact dystrophin-deficient mdx muscle fibres [17] are reported.

This study was undertaken in order to follow in mdx mice the release of two enzymes, both of cytoplasmic origin in different conditions. The rationale behind the choice of creatine kinase and lactate dehydrogenase determinations was the fact of their different molecular weights, and also their different activities in serum in the course of human dystrophy. The rationale behind the choice of different types of muscles of the mdx animals were the drastic differences in the expression of dystrophic changes in skeletal muscles and diaphragm, observed as degradation and active regeneration in skeletal muscles, and more human dystrophy-like changes in the mdx diaphragm. Because of the evident relationship of the morphological changes, at least, and the age of the animals the examinations were performed at the age between 14 and 360 days (at the pre necrotic stage in young and the degenerative-regenerative stages in adults), without and in the presence of different agents affecting the intracellular calcium concentration and/or its distribution, as high K^+ concentration, calcium ionophore A23187, Dantrolene and Verapamil. High K^+ is known to depolarize the muscle membrane, it induces a release of Ca^{2+} from sarcoplasmic reticulum into the cytoplasm, and increases protein degradation [42]. Calcium ionophore A23187 acts on the outer muscle membrane and causes a large increase in whole tissue calcium content [15, 21, 33, 35], it increases also LDH release from rats diaphragm [35], intensifies excitation-contraction coupling by potentiating Ca^{2+} intracellular stores release [6], induces membrane changes including depolarization with normal sensitivity to ouabain and alters the active sodium transport [37], enhances also protein degradation [12, 33, 42] and provokes dissolution of myofilaments [7]. Dantrolene impairs excitation-contraction coupling, protects the muscle against exercise-induced damage [1]. Its major action is an inhibition of calcium release from sarcoplasmic reticulum [16], it also acting on sarcolemma [40]. Verapamil has been shown to inhibit a voltage-dependent component of calcium influx in the cell membrane and transverse tubules [13] and causes also significant decrease of proteolysis [42].

Some of these agents were already used either in treating Duchenne dystrophy patients or applied to different dystrophy models both in vivo, as well as in vitro. In the in vitro studies with ionophore A23187 induced a significant increase of proteolysis of both normal and mdx mice muscles [24]. The calcium ionophore induces also rapid creatine kinase efflux but of the same magnitude from mdx and control muscles [25]. Chronic administration of Dantrolene to mdx mice lowers their serum creatine kinase

activity [32].

The presented data indicate that there was a substantial release in vitro predominantly of creatine kinase. Creatine kinase release was significant from both types of muscles in adult mdx animals. A lower CK release from skeletal muscles of young mdx animals is also reported by others [27]. A significant release of LDH from skeletal muscles was also age-dependent, being present only at the top of the activity of degeneration and regeneration processes. The release of both enzymes was augmented especially from mdx diaphragm, by agents provoking calcium accumulation, less when calcium distribution changes were induced. The former finding is contrary to the previously reported [25]. The enzymes efflux was less pronounced in young mdx animals than in the adults, as well as controls.

Blocking the calcium entry and/or improving its intracellular distribution was more effective in reducing the enzymes release in normals than in the mdx. In the latter, the reaction was age-dependent, more effective in young mdx animals, less in the mdx adults.

The degree of the enzymes efflux in young mdx animals would indicate that an increase of muscle membrane permeability is not a primary pathogenic effect of dystrophin deficiency in the muscle cells, as it is already suggested [27]. The fact, however, that calcium ionophore, Dantrolene and Verapamil are acting on the enzymes efflux in a different way as compared to normals even in young animals, would indicate that subtle muscle membrane permeability changes are present already at the pre necrotic stage of the disease.

Although there are several limitations in treating the mdx mice as a true Duchenne dystrophy model we hope that these observation may prove useful in developing new means to normalize one of the characteristic dystrophy symptoms evidenced as high enzymes release from the dystrophic muscle cell.

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References

- [1] Amelink GJH, Van der Kallen CJH, Wokke JHJ, Dop Bar PR: Dantrolene sodium diminishes exercise-induced muscle damage in the rat. *Eur J Pharmacol* 1990; 179: 187-192.
- [2] Bertorini TE, Palmieri GMA, Griffin JW, Igarashi M, Mc Gee J, Brown R, Nutting DF, Hinton AB, Karas JG: Effect of chronic treatment with the calcium antagonist diltiazem in Duchenne muscu-

- lar dystrophy. *Neurology* 1988; 38: 609-613.
- [3] Bhattacharya YK, Palmieri GMA, Bertorini TE, Nutting DF: The effect of diltiazem in dystrophic hamsters. *Muscle Nerve* 1982; 5: 73-78.
- [4] Bodensteiner JB, Engel AG: Intracellular calcium accumulation in Duchenne dystrophy and other myopathies: a study of 567,000 muscle fibers in 114 biopsies. *Neurology* 1978; 28: 439-446.
- [5] Brooke MH, Fenichel GM, Griggs RC, Mendel JR, Miller JP, Moxley R: A trial of nifedipine in Duchenne muscular dystrophy. *Neurology* 1984; 34 (Suppl. 1): 290.
- [6] Desmedt JE, Hainaut K: The effect of A23187 ionophore on calcium movements and contraction processes in single Barnacle muscle fibres. *J Physiol* 1976; 257: 87-107.
- [7] Duncan CJ: Role of intracellular calcium in promoting muscle damage: a strategy for controlling the dystrophic condition. *Experientia* 1978; 34: 1531-1672.
- [8] Duncan CJ: Dystrophin and the integrity of the sarcolemma in Duchenne muscular dystrophy. *Experientia* 1989; 45: 175-177.
- [9] Emery AEH, Skinner R, Howden LL, Matthews MB: Verapamil in Duchenne muscular dystrophy. *Lancet* 1982; ii: 559.
- [10] Engel WK: Integrative histochemical approach to the defect of Duchenne muscular dystrophy, in Rowland LP (ed): *Pathogenesis of Human Muscular Dystrophies*. Amsterdam, Oxford, Excerpta Medica, 1977, pp 277-309.
- [11] Franco A, Lansman JB: Calcium entry through stretch-inactivated ion channels in mdx myotubes. *Nature* 1990; 334: 670-673.
- [12] Furuno K, Goldberg AL: The activation of protein degradation in muscle by Ca^{2+} or muscle injury does not involve a lysosomal mechanism. *Biochem J* 1986; 237: 859-864.
- [13] Galizzi J-P, Fosset M, Lazdunski M: Properties of receptors for the Ca^{2+} -channel blocker verapamil in transverse-tubule membranes of skeletal muscle. Stereospecificity, effect of Ca^{2+} and other inorganic actions, evidence for the two categories of sites and effect of nucleoside triphosphates. *Eur J Biochem* 1984; 144: 211-215.
- [14] Glesby MJ, Rosenmann E, Nylén G, Wrongemann K: Serum CK, calcium, magnesium, and oxidative phosphorylation in mdx mouse muscular dystrophy. *Muscle Nerve* 1988; 11: 852-856.
- [15] Grinstein S, Erlj D: Action of insulin and cell calcium - Effect of ionophore A23187. *J Membr Biol* 1976; 29: 313-328.
- [16] Hainaut K, Desmedt JE: Effect of Dantrolene sodium on calcium movement in single muscle fibres. *Nature* 1974; 252: 728-730.
- [17] Head SI: Membrane potential, resting calcium and calcium transients in isolated muscle fibres from normal and dystrophic mice. *J Physiol* 1993; 469: 10-11.
- [18] Hoffman EP, Brown RH, Kunkel LM: Dystrophin: the protein product of the Duchenne muscular dystrophy locus. *Cell* 1987; 51: 919-928.
- [19] Hoffman EP, Knudson CM, Campbell KP, Kunkel LM: Subcellular fractionation of dystrophin to the triads of skeletal muscle. *Nature* 1987; 330: 754-758.
- [20] Jones DA, Jackson MJ, Edwards RTH: Release of intracellular enzymes from an isolated mammalian skeletal muscle preparation. *Clin Sci* 1983; 65: 193-201.
- [21] Kameyama T, Etlinger JD: Calcium-dependent regulation of protein synthesis and degradation in muscle. *Nature* 1979; 279: 344-346.
- [22] Koenig M, Monaco AP, Kunkel LM: The complete sequence of dystrophin predicts a rod-shaped cytoskeletal protein. *Cell* 1988; 53: 219-228.
- [23] Lowry OH, Rosebrough NJ, Farr AE, Randall RJ: Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; 193: 265-275.
- [24] Mac Lennan PA, Mc Ardle A, Edwards RHT: Effects of calcium on protein turnover of incubated muscle from mdx mice. *Am J Physiol* 1991; 260: E 594-E 598.
- [25] Mc Ardle A, Edwards RTH Jackson MJ: Effects of contractile activity on muscle damage in the dystrophin-deficient mdx mouse. *Clin Sci* 1991; 80: 367-371.
- [26] Mc Ardle A, Edwards RTH Jackson MJ: Accumulation of calcium by normal and dystrophin-deficient muscle during contractile activity "in vitro". *Clin Sci* 1992; 82: 455-459.
- [27] Mc Ardle A, Edwards RTH Jackson MJ: Time course of changes in plasma permeability in the dystrophin-deficient mdx mouse. *Muscle Nerve* 1994; 17: 1378-1384.
- [28] Monaco AP, Bertelson CJ, Middlesworth W, Colletti CA, Aldridge J, Fischbeck KH, Bartlett R, Pericak-Vance MA, Roses AD, Kunkel LM: Detection of deletions spanning the Duchenne muscular dystrophy locus using a tightly linked DNA segment. *Nature* 1985; 316: 842-845.
- [29] Moxley RT, Brooke MH, Fenichel GM, Mendel JR, Griggs RC, Miller JP, Province MA, Patterson V, and the CIDD Group: Clinical investigation in Duchenne dystrophy. VI Double-blind controlled trial of nifedipine. *Muscle Nerve* 1987; 10: 22-33.
- [30] Pernice WR, Beckmann R, Ketelsen U-P, Frey B, Schmidt-Redemann KP, Haap R, Rochren R, Sauer

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- M: A double-blind placebo controlled trial of diltiazem in Duchenne dystrophy. *Klin Wschr* 1988; 66: 565-570.
- [31] Peterson GL: A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal Biochem* 1977; 83: 346-356.
 - [32] Quinlan IG, Johnson SR, Samaha FJ: Dantrolene normalizes serum creatin kinase in mdx mice. *Muscle Nerve* 1990; 13: 268-269.
 - [33] Rodemann HP, Waxman L, Goldberg AL: The stimulation of protein degradation in muscle by Ca^{2+} is mediated by prostaglandin E_2 and does not require the calcium-activated protease. *J Biol Chem* 1982; 25: 8716-8723.
 - [34] Soybel D, Morgan J, Cohen L: Calcium augmentation of enzyme leakage from mouse skeletal muscle and its possible site of action and pharmacology. *Res Comm Chem Path* 1978; 20: 317-329.
 - [35] Sudgen PH: The effects of calcium ions, ionophore A23187 and inhibition of energy metabolism on protein degradation in the rat diaphragm and epitrochlearis muscles in vitro. *Biochem J* 1980; 190: 593-603.
 - [36] Takagi A, Kojima S, Ida M, Araki M: Increased leakage of calcium ion from the sarcoplasmic reticulum of the mdx mouse. *J Neurol Sci* 1992; 110: 160-164.
 - [37] Takamori M, Ide Y, Tsujikata M: Pharmacophysiological study on muscle treated with calcium ionophore A23187. *J Neurol Sci* 1981; 50: 89-94.
 - [38] Turner PR, Westwood T, Regen CM, Steinhardt RA: Increased protein degradation results from elevated free calcium levels found in muscle from mdx mice. *Nature* 1988; 335: 735-738.
 - [39] Turner PR, Fong P, Denetclaw WF, Steinhardt RA: Increased Ca^{2+} influx in dystrophic muscle. *J Cell Biol* 1991; 115: 1701-1712.
 - [40] Ward A, Chaffman O, Sorkin EM: Dantrolene - A review of its pharmacodynamic and pharmacokinetic properties and therapeutic use in malignant hypertermia, the neuroleptic malignant syndrome and an update of its use in muscle spasticity. *Drugs* 1986; 32: 130-134.
 - [41] Zalman F, Perloff JK, Durant NN, Campion DS: Acute respiratory failure following intravenous verapamil administration in Duchenne's muscular dystrophy. *Amer Heart J* 1983; 105: 10-11.
 - [42] Zeman RJ, Kameyama T, Matsumoto K, Bernstein P, Etlinger JD: Regulation of protein degradation in muscle by calcium. Evidence for enhanced nonlysosomal proteolysis associated with elevated cytosolic calcium. *J Biol Chem* 1985; 260: 13619-13624.