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Activity of sarcoplasmic and mitochondrial creatine kinase in mouse muscular dystrophy: effect of early immobilization

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Abstract

The abnormal distribution of energy-rich phosphates in dystrophic muscle gave rise to compare the sarcoplasmic and mitochondrial creatine kinase (EC 2.7.3.2) activity in healthy and dystrophic muscle. In adult three month old dystrophic mice (129 ReJ, dy/dy) both the sarcoplasmic and mitochondrial creatine kinase activity in the muscles of the lower hind legs was only about 30 % compared to healthy controls (dY/dY).

As judged by histological parameters, immobilization of the hind legs of dystrophic mice during the second week of life is known to slow down the progression of the disease. Therefore we examined the effect of early immobilization on the creatine kinase activity. It could be shown that in adult dystrophic mice the creatine kinase activity in muscles from the left hind leg that has been immobilized during the second week of life was about 1.5-fold higher then the activity in the muscles from the right hind leg that has not been immobilized. It is concluded that the abnormal distribution of energy-rich phosphates in dystrophic muscle is not specifically linked to a decrease of either the sarcoplasmic or mitochondrial creatine kinase activity. In addition to histological parameters, it could now also be shown by a biochemical parameter of muscular dystrophy that progression of the disease can be slowed down by a restricted use of the muscles in an early stage of life.

Keywords: creatine kinase, muscular dystrophy, mouse, early immobilization

Introduction

Muscular dystrophy is a heterogeneous group of hereditary diseases in both humans and animals with muscular weakness due to fiber necrosis as a common feature. Little is known about the primary biochemical changes that initiate a cascade resulting in muscle damage, with features as: fibrosis, changed membrane permeability, fatty infiltration.

As a relative new feature Wirtz et al. (1987) were able to show an abnormal distribution of energy-rich phosphates in the dystrophic muscle of mice by using the 'in

vivo ^{31}P NMR' technique. This feature focusses the attention to the enzyme creatine kinase (CK; EC 2.7.3.2) which plays a central role in the metabolism of energy-rich phosphates within the muscle. The enzyme is located at places where ATP is produced or consumed. When located in the mitochondria CK catalyzes the transfer of energy-rich phosphates to the phosphate-carrier creatine in order to form creatine phosphate. Located at the ATP-using sides (e.g. myofibril, Calcium-pump), CK is involved in the formation of ATP by a transfer of the energy-rich phosphate group from creatine phosphate to ADP. In total there is a creatine phosphate shuttle which serves as an initial short-term energy source for the muscle. CK has different isoforms at the different locations within the muscle. The mitochondrial CK has been separated from the sarcoplasmic CK by electrophoresis (e.g. Blum et al., 1981).

In the first part of this study we have compared the sarcoplasmic and mitochondrial creatine kinase activity in the hind leg muscles of adult healthy and dystrophic mice. In the second part of this study we have examined whether immobilization of the hind leg during the second week of life has any effect on the CK-activity in the muscles of adult dystrophic mice. Wirtz et al. (1986), using histological parameters to describe the severeness of the disease, have found that early immobilization of the hind leg of the dystrophic mice during their second week of life slows down the progression of the disease.

Materials and methods

Animals

Thirteen dystrophic mice (dy/dy) and 8 healthy control animals (dY/dY) of the Harbor strain 129 ReJ were used. Six of the dystrophic mice participated in the early immobilization experiment in which the left hind leg of the mice had been immobilized during the second week of life as described elsewhere (Wirtz et al., 1986). Muscle samples were taken when the mice were about 3 months old (98 d, SD 20).

Preparation of the samples

After the animals were killed by an overdose of ether, the muscles of the lower hind legs were dissected. In the immobilization experiment muscles of the left and right lower hind leg were separately prepared for the determination of CK-activity. Sample preparation was performed at 0 °C. The aim is to separate mitochondrial CK from sarcoplasmic CK.

Muscle tissue was carefully cutted to small pieces using a scalpel and, after adding SETH-medium (250 mM sucrose, 2 mM EDTA, 10 mM Tris-buffer, 50 U ml^{-1} heparin) homogenized with a potter Elvehjem homogenizer (clearance 40 μm). The amount of SETH added was 10 times the weight of the muscle tissue. Careful homogenizing was necessary in order to prevent destruction of the mitochondria and to avoid mixing mitochondrial CK with sarcoplasmic CK (Grace et al., 1983).

The mixture was centrifuged for 10 minutes, 600 $\times$ g at 0 °C. The pellet was discarded. Supernatant (1), with sarcoplasma and mitochondria was centrifuged dur-

ing 15 minutes, 14000 × *g* at 0 °C in order to separate mitochondria (lysosomes and microsomes) in pellet (2) from sarcoplasma in supernatant (3). The pellet (2) was washed four times: pellet was resuspended in 5 volumes (w w^{-1}) SETH and centrifuged (15 minutes, 14000 × *g*, 0 °C). Supernatants were discarded. Finally the pellet was suspended in 5 volumes (w w^{-1}) SETH and, together with supernatants (1 and 3) stored at −20 °C, until CK-activity was measured.

CK-activity measurements

The activity of creatine kinase was measured with creatine phosphate. The ATP formed reacted in the hexokinase reaction and the product glucose-6-phosphate in the glucose-6-phosphate dehydrogenase reaction. The production of NADPH was measured as an increase in extinction with isothermic spectrophotometry at 340 nm and 30 °C. The procedure was carried out as prescribed for the CK-reagents from Technicon (manual method).

Results

In the first part of this study we have compared the sarcoplasmic and mitochondrial creatine kinase activity in the hind leg muscles of adult healthy and dystrophic mice. The values are shown in Table 1. In adult three month old dystrophic mice both the sarcoplasmic and mitochondrial creatine kinase activity in the muscles of the lower hind legs was only about 30 % compared to healthy controls. In this study the contribution of mitochondrial CK to the total CK-activity is estimated to be about 0.03 %.

In the second part of this study we have examined whether immobilization of the hind leg during the second week of life has any effect on the CK-activity in the muscles of adult dystrophic mice. The individual values for 6 mice are shown in Table 2. Data show that as a result of early immobilization the decrease in the activity in

Table 1. Creatine kinase activity (U ml^{-1})[a] in hind leg muscles of adult 3 month old mice; Student's *t*-test.

	Sarcoplasmic + mitochondrial	Sarcoplasmic	Mitochondrial[c]
Control ($n=8$)	386[b]	448[b]	0.17
SD	60	62	0.07
Dystrophic ($n=7$)	126	130	0.04
SD	21	21	0.02
Decrease	67 %	71 %	74 %
P-value	(0.025)	(0.01)	(0.001)

[a] U = units enzyme activity (amount of enzyme [mg], converting 1 μmol substrate per minute, under constant pH and temperature).
[b] Student's *t*-test, $P = 0.1$, non-significant.
[c] Dystrophic: $n = 6$, instead of 7.

Table 2. Creatine kinase activity in left (L) and right (R) hind leg muscles of adult 3 month old mice (U ml^{-1}); Student's *t*-test, paired. The left hind leg has been immobilized during the second week of life.

Dystrophic mice ($n=6$)	Sarcoplasmic + mitochondrial		Sarcoplasmic		Mitochondrial	
	L	R	L	R	L	R
1	154.3	121.2	128.0	107.1	–	–
2	141.5	78.8	125.4	77.2	0.065	0.020
3	123.8	87.8	109.3	90.0	0.028	0.012
4	155.3	141.5	183.3	138.3	0.098	0.028
5	151.1	110.9	132.8	98.1	0.106	0.036
6	186.5	170.4	205.8	157.6	0.097	0.052
P-value	(0.005)		(0.005)		(0.005)	
Mean	152	118	147	111	0.08	0.03
SD	19	34	38	30	0.03	0.01

the left hind leg is significantly less compared to the contralateral hind leg. This effect is shown in both sarcoplasmic and mitochondrial CK-activity.

Discussion

In this study the sarcoplasmic and mitochondrial CK-activity of dystrophic muscle was about 30 % of the CK-activity in the muscles of healthy controls. Comparable results are found in other studies. Petell et al. (1984) found a 55-60 % decrease of CK-activity in mouse muscular dystrophy. In human studies a decrease in CK-activity in muscular dystrophy of 20 % (Chi et al., 1987) and 50-60 % (Scholte et al., 1980) is found.

It is assumed that the decrease in CK-activity is related to the severeness of the muscle damage. Not only CK, but the activity of many other muscle enzymes is decreased. Especially the glycogenolytic enzymes are decreased in activity 35-50 % (Petell et al., 1984) or even 90 % (Garber et al., 1980) measured in mice.

The contribution of mitochondrial CK-activity to total CK-activity in this study is about 0.03 %. In human studies Apple & Rogers (1986) found that the percentage of mitochondrial CK activity in human muscle is <1 %. Walliman & Eppenberger (1985) measured a 5 % mitochondrial CK-activity and Bessman & Carpenter (1985) $>>5$ %.

This study shows that there is no specific link between the decrease of total CK-activity and the sarcoplasmic or mitochondrial iso-enzyme. This may indicate an overall deterioration of the creatine-phosphate shuttle or a decrease in other enzymes and compounds needed for muscle function.

The second experiment further supports that due to early immobilization, the CK-activity in dystrophic muscles is found to decrease in a lesser extend. Early immobilization and denervation of dystrophic muscles in mice (129 ReJ) is seen to reduce muscle damage (Wirtz et al., 1986; Jaros & Johnstone, 1983). A greater number of

functional muscle fibers was observed, and the mice were able to make better use of their remobilized hind leg compared to the contralateral hind leg as a result of the early immobilization. Immobilization has a muscle sparing action in murine dystrophy.

It could be possible that the contralateral muscles are additionally damaged during immobilization as a result of the extra load. However the microscopic picture of the contralateral muscles did not differ from dystrophic muscles of mice, which were not immobilized (Wirtz et al., 1986).

The use of the muscle seems to stimulate the progression of the disease. This is consistent with the hypothesis of Wrogemann et al. (1979): a membrane defect causes leaking of calcium into the cell causing high sarcoplasmic calcium concentrations and mitochondrial dysfunction, due to increasing calcium concentrations within the mitochondria. The high calcium concentrations cause hypercontractions leading to energy depletion and cell death. The use of the muscle seems to accelerate this process.

Besides histological studies, muscle CK-activity can be used as parameter for the progression of muscular dystrophy.

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References

Apple, F. S. & M. A. Rogers, 1986. Mitochondrial creatine kinase activity alterations in skeletal muscle during long distance running. *American Physiological Society* 61: 482-485.

Bessman, S. P. & C. L. Carpenter, 1985. The Creatine-Creatinephosphate-shuttle. *Annual Reviews of Biochemistry* 54: 831-862.

Blum, H. E., B. Weber, B. Deus & W. Gerol, 1981. The mitochondrial creatine kinase isoenzyme from human heart muscle. In: H. Lang (Ed.), Creatine kinase isoenzymes, pathophysiology and clinical application, p. 19-30. Springer-Verlag, New York.

Chi, M. M., C. S. Hintz, D. McKee, S. Felder, N. Grant, K. K. Kaiser & O. H. Lowry, 1987. Effects of duchenne dystrophy on enzymes of energy metabolism in individual muscle fibers. *Metabolism* 36: 761-767.

Garber, A.J., L. Birnbaumer, E. P. Bornet, W. J. Thompson & M. L. Entman, 1980. Skeletal muscle protein and amino acid metabolism in hereditary mouse muscular dystrophy (II). *Journal of Biological Chemistry* 255: 8325-8333.

Grace, A. M., M. B. Perryman & R. Roberts, 1983. Purification and characterization of human mitochondrial creatine kinase. *Journal of Biological Chemistry* 258: 15346-15354.

Jaros, E. & D. Johnstone, 1983. Effect of denervation upon muscle fiber number in normal and dystrophic (dy/dy) mice. *Proceedings of the Physiological Society* 343: 104-105.

Petell, J. K., N. A. Marshall & H. G. Lebherz, 1984. Content and synthesis of several abundant glycolytic enzymes in skeletal muscles of normal and dystrophic mice. *International Journal of Biochemistry* 16: 61-67.

Scholte, H. R., H. F. M. Busch, I. E. M. Luyt-Houwen, J. T. Stinis, F. G. I. Jennekens & W. Mortier, 1980. Muscle carnitine in DMD. In: C. Angelini, G. A. Danieli, D. Fontanari (Eds), Muscular dystrophy research advances and new trends. Exerpta Medica, Amsterdam: 303-304.

Walliman, T. & H. M. Eppenberger, 1985. Localization and function of M-line bound creatine kinase. In: W. Shay (Ed.), Cell and muscle motility, Vol. 6, p. 239-285. Plenum Publishing Corp., New York.

Wirtz, P., H. Loermans & W. Wallinga-de Jonge, 1986. Longterm functional improvement of dystrophic mouse leg muscles upon early immobilization. *Britisch Journal of Experimental Pathology* 67: 201-208.

Wirtz, P., H. Loermans, J. Joordens & C. Hilbers, 1987. In vivo 31-P-NMR spectroscopy applied to mice with muscular dystrophy. Proceedings of the Dutch Federation Meeting, Nijmegen, p. 551.

Wrogemann, K., W. A. K. Hayward & M. C. Blanchaer, 1979. Biochemical aspects of muscle necrosis in hamster dystrophy. In: J. Harris (Ed.), Muscular dystrophy and other inherited diseases of skeletal muscle in animals. *Annals of the New York Academical Sciences* 317: 30-45.

This synopsis is based on a student thesis entitled 'Metabolisme van sarcoplasmatische eiwitten by de dystrofe muis: het enzym creatine kinase' by Annet Roodenburg, Department of Human and Animal Physiology, Wageningen Agricultural University, Wageningen, 1988, 46 pp., 31 figs, 4 tables, 44 refs, 5 apps, in Dutch. Available as paper copy (order R108, f 10,– including postage) at NARD, c/o Pudoc, P.O. Box 4, 6700 AA Wageningen (telex 45015 bluwgnl).
