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Research Articles

Dystrophin-dependent efficiency of metabolic pathways in mouse skeletal muscles

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Abstract. Muscles from the *mdx* mouse (X-linked genetic disorder similar to Duchenne muscular dystrophy) lack dystrophin-associated transsarcolemmal proteins[1] and show reduced maintenance metabolic rates[2]. Here, microcalorimetric comparisons of metabolic stimulation by exogenous substrates in isolated muscles revealed substrate-selective limitation of chemical reaction rates through both glycolytic and TCA-cycle pathways, identical in slow- and fast-twitch *mdx* muscles. This systemic approach, as opposed to comparisons of single-enzyme activities, sheds new light on the function of dystrophin and associated proteins. The in vivo efficiency of metabolic pathways may depend on stabilization of enzyme complexes by dystrophin-associated elements of the cytoskeleton.
Key words. Muscular dystrophy; skeletal muscle; energy metabolism; subcellular compartmentation; substrate channelling; cytoskeleton; dystrophin.

The energy status of skeletal muscle cells is subnormal in Duchenne/Becker muscular dystrophy[3-5] and its animal models, including the *mdx* mouse[6]. This long-known observation has been overshadowed by the findings of increased intracellular Ca^{2+} and abnormal permeation of Ca^{2+} through the sarcolemma[7-9] in these tissues. A putative high energy demand for sarcoplasmic Ca^{2+} homeostasis was thought to be the cause of the low energy status. However, a newly developed microcalorimetric approach to assess the rate of energy expenditure associated with Ca^{2+} recirculation between sarcoplasmic reticulum and sarcoplasm[10] revealed this to be lower by 62% in soleus muscles and 29% in extensor digitorum longus (EDL) muscles in adult *mdx* mice as compared with corresponding muscles from control C57Bl/10 mice[2]. Together with evidence that the rate of other energy-consuming processes is also decreased, this suggested that the lack of dystrophin may cause some primary deficiency in energy conversion in *mdx*-mouse muscle cells when at rest (Even et al., unpublished) or when metabolically active[11,12].

Comparisons of the maximum catalytic activities of single enzymes in *mdx* or control muscle fibres failed to reveal any precise metabolic defect in the former, but multivariate quantitative enzyme histochemistry enabled Stoward and Stapleton to distinguish dystrophic from normal fibre samples[13]. Evidence of specific glycolytic enzyme associations to mitochondrial outer membrane and actin or actin-related protein microfilaments[14-18], association of other cytoskeletal elements to mitochondria[19-28] (schematized in fig. 1), and the greater efficiency of mitochondrial biochemical pathways in situ – whether the TCA cycle or the respiratory chain – as compared to in isolated organelles[29-31], are all compatible with the idea that dystrophin might associate with metabolic enzyme complexes and, thereby, determine the over-all efficiency of metabolic pathways.

In this work we analysed metabolic pathways in situ by imposing large changes of substrate availability on superfused, glucose-derived muscles. This way of modulating cellular energy production by substrate oxidation might be considered an extension to intact tissue-cell preparations of the procedure used by Krebs and Johnson on diluted suspensions of minced pigeon breast muscle when, in 1937, they first disclosed the role of a four-carbon dicarboxylic acid as a carrier molecule in the oxidation of a 'triose'[32]. This procedure is remarkably efficient in mammalian muscles, which quickly readjust their rate of maintenance energy expenditure when exposed to an exogenous substrate[33]. The excess of converted energy is dissipated at the same rate, as a consequence of in situ stimulation of proton recirculation through inner mitochondrial membranes and of various ATP-dependent processes[34]. Figure 2 shows an example of the reversible thermogenic responses to various substrates measured in soleus muscles preequilibrated for 3 h in a purely electrolytic environment, under conditions of mechanical restraint, oxygenation and temperature that preserve muscle function[10].

Intuitively, the change in the chemical flux through a 'channelled' metabolic pathway upon alteration of an environmental effector molecule (for example a substrate, or a carrier molecule in a cycle of reactions) would be expected to be larger than that through the

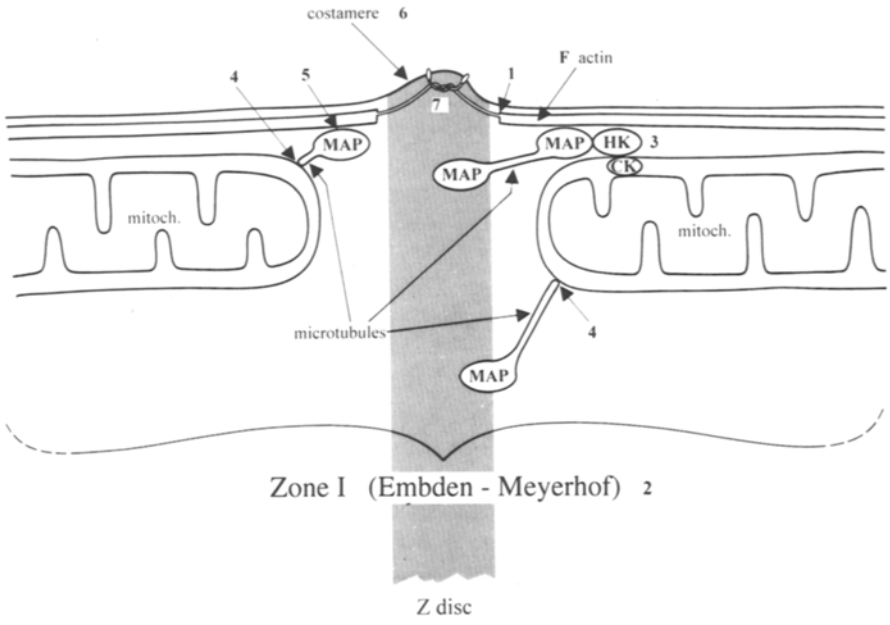

Figure 1. Schematic representation of possible interactions of dystrophin with microtubules, via F actin and microtubule-associated proteins (MAP), or through Z discs' edges corresponding to regions of dystrophin accumulation[51–54]: *1* attachment of dystrophin (*7*) to F actin; *2* location of enzymes of the Embden-Meyerhof pathway at the site of the actin filaments within the I zone; *3* attachment of hexokinase (HK) to outer mitochondrial membrane, at sites of phosphocreatine transfer to sarcoplasm after phosphorylation by mitochondrial creatine kinase (CK); *4* attachment of microtubules to mitochondria; *5* possible interaction of MAP with dystrophin (*7*) via F actin; *6* possible site of interaction of Z-disc MAP with dystrophin (*7*).

corresponding non-channelled pathway. This is supported by recent theoretical demonstrations[35,36]. There is no contradiction here with the fact that maximum activities in vitro of enzymes that catalyze near-equilibrium reactions in situ are considerably higher than the maximum flux through the pathway to which they belong. In vitro a single enzymatic reaction is tested, while in situ the chemical flux through the whole pathway will remain limited by the non-equilibrium, control reactions. To test the degree of channelling of glycolysis and oxidative metabolism in *mdx* vs control muscles, we measured the metabolic responses to the input substrate (glucose) and the last product (pyruvate) of glycolysis, and to two TCA-cycle intermediates, citrate and succinate. We also tested fumarate and acetylene dicarboxylate, the carrier molecule of a cyclic reaction sequence proposed as the most direct route for the in situ oxidative decarboxylation of enol pyruvate[37]. We now present the results and discuss how, together with previous findings, they point to some primary loss of efficiency of the TCA cycle and of glycolysis in *mdx*-mouse muscles.

The response to glucose but not to succinate was markedly attenuated in *mdx* muscles (see fig. 2; table 1, rows 1 and 4). Since the rate of glucose uptake by *mdx* muscles was normal (table 2), the thermogenic response to glucose confirms that a decreased proportion of the glucose is oxidized in muscles from *mdx* mice[38]. This is also in keeping with observations of marginal deficits in mitochondria- and cytoskeleton-bound glycolytic enzymes[39,40].

The results with pyruvate (table 1, row 2) tend to confirm the observation that *mdx* muscle mitochondria have a reduced ability to handle this substrate[41] (the

Table 1. Sustained thermogenic responses (i.e., total minus basal heat production rate) of isolated slow-twitch (soleus) and fast-twitch (EDL) muscles from control and *mdx* mice to substrates added to the superfusion saline.

	Soleus control	*mdx*	EDL control	*mdx*
Glucose 5 mM	0.71 ± 0.11	0.22 ± 0.02	0.34 ± 0.04	0.07 ± 0.02
Pyruvate 10 mM	2.59 ± 0.15	1.05 ± 0.06	2.07 ± 0.10	1.39 ± 0.07
Acetylene dicarboxylate 10 mM	2.25 ± 0.12	1.23 ± 0.11	2.14 ± 0.14	1.01 ± 0.08
Succinate 10 mM	2.03 ± 0.17	1.82 ± 0.11	0.81 ± 0.13	1.10 ± 0.10
	NS		NS	
Fumarate 10 mM	0.73 ± 0.09	0.78 ± 0.05	0.33 ± 0.07	0.47 ± 0.07
	NS		NS	

Mean values (mW/g w. wt.) ± SEM ($n = 13–15$ for pyruvate and succinate; $n = 7$ for other substances). Except for those marked NS, differences are all statistically significant ($p < 0.01$).

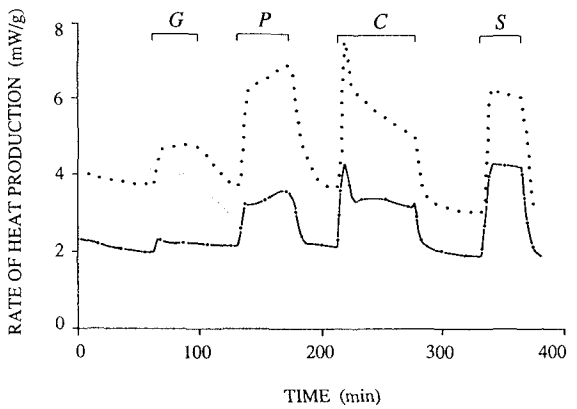

Figure 2. Thermogenic responses of control (. . .) and *mdx* (__) soleus muscle pairs to 5 mM glucose (G), 10 mM pyruvate (P), citrate (C) and succinate (S) successively added to the superfusate for 40 to 60-min periods. The rate of heat production was expressed in mW per gram of blotted muscle weight measured after removal of the tendons. The low basal value of the *mdx* pair is only partly the consequence of a low energy cost of intracellular Ca^{2+} homeostasis[2]. The most striking difference between *mdx* and control (here slow-twitch muscles but, as shown in table 1, also fast-twitch ones) in their response to substrates was the relatively smaller response of *mdx* muscles to glucose or to pyruvate, as opposed to their relative response to succinate (which was larger in the *mdx* muscle pair).
Methods. Animals were raised and kept in accordance with 'Good Laboratory Practice' guidelines, and received unlimited water and food. After decapitation of the animals, muscles were dissected out and freed from loosely attached connective tissue without the instruments touching the fibres and then each pair was immediately held at about 130% of its relaxation length on a stainless-steel frame, in the test chamber of a twin heat-flux microcalorimeter perfused at 30 °C with a Krebs-Ringer bicarbonate solution filter-sterilized and equilibrated on-line with a thin-layer tonometer at mean O_2 and CO_2 partial pressures of 85 and 5.3 kPa, respectively. The calorimetric signal, proportional to total heat production rate by a pair of muscles, was equal to the voltage difference between two series of six thermal gradient layers surrounding test and control chambers minus a blank difference recorded before introduction of the muscles into the test chambers[10].

stronger stimulatory effect of exogenous pyruvate than of glucose on muscle energy conversion in general has been recently discussed by others[42,43]). Further testing, with fumarate, of substrate channelling in the succinyl-CoA synthetase to malate dehydrogenase span of the TCA cycle[30] revealed no difference between *mdx* and control muscles (table 1, row 5), though fumarate produced a smaller response than succinate (which has also been observed with morphologically and biochemically intact muscle mitochondria[44]).

Acetylene dicarboxylate, the unconventional four-carbon intermediate, induced a significant stimulation of energy metabolism which was substantially larger in normal than in *mdx* muscles. In fact, the enhancement of energy metabolism by acetylene dicarboxylate was reduced by about 50% in both types of *mdx* muscles, as was the response to the substrate of the overall reaction (table 1, rows 2 and 3). This new result supports the idea that the as yet hypothetical 'one-step reaction' form of the TCA cycle is indeed the physiological cycle in situ, and that the lack of dystrophin causes deficiencies in both glycolysis *and* the TCA cycle.

At least two alternative interpretations of the data, however, are possible. First, as suggested by the quantitative difference between the defects of glucose and pyruvate utilization by heart muscles from the hamster model of X-linked muscle dystrophy[45], or in the present experiments by *mdx*-mouse skeletal muscle (about 70% and 50% less than control for glucose and pyruvate, respectively), the decrease in mitochondrial activity might only be due to glycolytic inhibition. The non-soluble form of hexokinase II, like glycerol kinase and contrary to all other glycolytic enzymes[16,46], does not bind to the cytoskeleton proper but rather interacts with the outer surface of the outer mitochondrial membrane where it might have preferential access to the energy produced by oxidative phosphorylation, via the adenine nucleotide translocator of the inner membrane, mitochondrial creatine kinase and the outer-membrane protein porin in its anion-selective state[46,47]. Since in X-linked dystrophies isoenzymes of hexokinase II[48] differ physicochemically from the normal enzyme, one may speculate that the above-mentioned functional link between an ATP-consuming reaction in the cytoplasm and mitochondrial energy production is compromised and the efficiency of the TCA cycle decreased. Such an interpretation is in keeping with our observation that

Table 2. Rate of glucose uptake by intact soleus and EDL muscles from *mdx* and control mice, in the absence and presence of insulin.

	Soleus control	*mdx*	EDL control	*mdx*
Basal	3.93 ± 0.63	5.01 ± 0.46	2.26 ± 0.57	2.52 ± 0.49
+Insulin 0.025 U/l	10.02 ± 0.92	13.08 ± 1.35	5.88 ± 0.53	4.96 ± 0.47

Results are expressed in mmol/h per g w. wt. (means ± SEM of 5 experiments). The differences between *mdx* and control were not statistically significant. Muscle preparations were identical to those for calorimetry experiments, and preincubated under the same conditions except that glucose (5 mM) was present. Then a first 20-min incubation took place in a 5-ml volume of Krebs-Ringer solution containing bovine serum albumin (0.2%) with or without insulin (25 mU/l), followed by a second 20-min incubation in a 2-ml volume of the same solution supplemented with 2 mCi of tritiated 2-deoxy-D-glucose. The muscles were rinsed 5 times in 5-ml volumes of ice-cold saline to remove extracellular marker, muscles were then separated from their tendons, blotted dry, weighed, dissolved in 0.5 ml BTS 450 (tissue solubilizer, Beckman) overnight at room temperature, added to 10 ml of a liquid scintillation mixture (Ready organic, Beckman) including 0.7% glacial acetic acid and counted with a Beckman SL 7500 counter. Glucose taken up during the last 20-min incubation period was quantified from calibrations obtained with the freshly prepared batch of the final incubation medium used in the experiment.

even under glucose deprivation, i.e., when the main energy fuel is endogenous triglycerides, maintenance energy dissipation is lower in *mdx* than in normal muscles. Glycerol kinase is the other cytoplasmic, mitochondria-bound enzyme which drives an ATP-consuming reaction[46]. However, our recent finding that octanoate, whose catabolism does not depend on glycolysis[49] and does not generate glycerol, is also less well utilized in *mdx* than in normal muscles (Even et al., unpublished) tends to invalidate this interpretation of the data.

A second alternative is that high intracellular Ca^{2+}, in dystrophic fibres, may be the cause of both glycolysis inhibition (by calmodulin-induced inactivation of phosphofructokinase[50]) and the inefficiency of mitochondrial metabolic pathways[51]. Mitochondrial overload with calcium could be such that the concentration of free Ca^{2+} in the matrix would exceed its well-defined 0.1–1 micromolar optimum for the physiological control of pyruvate, (NAD^+)-isocitrate and 2-oxoglutarate dehydrogenase activities on one hand, and of ATP synthase activity on the other[34]. Recent observations, however, seem to invalidate this second alternative too. Discrete potassium depolarizations of the sarcolemma produce significant, sustained increases of Ca^{2+}-dependent metabolic rate under conditions where the rise in sarcoplasmic Ca^{2+} is not high enough to induce contraction[10]. Contrary to basal and contraction-associated Ca^{2+}-dependent heat productions, these Ca^{2+}-dependent stimulations of metabolic rate can be as large in *mdx* as in normal muscle fibres[2].

The present results suggest that the efficiency of cytoplasmic and mitochondrial metabolic pathways in situ may rely on a dystrophin-dependent cytoskeletal organization in both slow- and fast-twitch skeletal muscles of the mouse. More generally, the lack of dystrophin (*mdx* mouse; Duchenne muscular dystrophy patient), or a functionally mildly defective dystrophin (female Duchenne/Becker muscular dystrophy carriers[11]), could be directly responsible for subnormal rates of muscle energy conversion and the subnormal energy status of sarcoplasm.

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1 Ervasti, J. M., and Campbell, K. P., Cell *66* (1991) 1121.
2 Decrouy, A., Even, P. C., and Chinet, A., Experientia *49* (1993) 843.
3 Vignos, P. J., and Lefkowitz, M., J. clin. Invest. *38* (1959) 873.
4 Younkin, D. P., Berman, P., Sladky, J., Chee, C., Bank, W., and Chance, B., Neurology *37* (1987) 165.
5 Barbiroli, B., Funicello, R., Lotti, S., Montagna, P., Ferlini, A., and Zaniol, P., J. neurol. Sci. *109* (1992) 188.
6 Dunn, J. F., Frostick, S., Brown, G., and Radda, G. K., Biochim. biophys. Acta *1096* (1991) 115.
7 Turner, P. R., Westwood, T., Regen, C. M., and Steinhardt, R. A., Nature *335* (1988) 735.
8 Fong, P., Turner, P. R., Denetclaw, W. F., and Steinhardt, R. A., Science *250* (1990) 673.
9 Franco, A., and Lansman, J. B., Nature *344* (1990) 670.
10 Chinet, A., Decrouy, A., and Even, P. C., J. Physiol. *455* (1992) 663.
11 Barbiroli, B., Funicello, R., Ferlini, A., Montagna, P., and Zaniol, P., Muscle & Nerve *15* (1992) 344.
12 Dunn, J. F., Tracey, I., and Radda, G. K., J. neurol. Sci. *113* (1992) 108.
13 Stoward, P. J., and Stapleton, A., Histochem. J. *19* (1987) 598.
14 Arnold, H., and Pette, D., Eur. J. Biochem. *6* (1968) 163.
15 Hofer, H. W., Gerlach, G., Birkel, G., and Kirschenlohr, H. L., Biochem. Soc. Trans. *15* (1987) 982.
16 Chen-Zion, M., Bassukevitz, Y., and Beitner, R., Int. J. Biochem. *24* (1992) 1661.
17 Minaschek, G., Gröschel-Stewart, U., Blum, S., and Bereiter-Hahn, J., Eur. J. Cell Biol. *58* (1992) 418.
18 Ervasti, J. M., and Campbell, K. P., Curr. Op. Cell Biol. *5* (1993) 82.
19 Griffith, L. M., and Pollard, T. D., J. Cell Biol. *78* (1978) 958.
20 Ball, E. H., and Singer, S. J., Proc. natl Acad. Sci. USA *79* (1982) 123.
21 Hirokawa, N., J. Cell Biol. *94* (1982) 129.
22 Arakawa, T., and Frieden, C., J. biol. Chem. *259* (1984) 11730.
23 Linden, M., Nelson, B. D., and Leterrier, J.-F., Biochem. J. *261* (1989) 167.
24 Price, M. G., and Gomer, R. H., Cell Motil. Cytoskel. *13* (1989) 274.
25 Gupta, R. S., TIBS *15* (1990) 415.
26 Saetersdal, T., Greve, G., and Dalen, H., Histochemistry *95* (1990) 1.
27 Kano, Y., Fujimaki, N., and Ishikawa, H., Cell Struct. Funct. *16* (1991) 251.
28 Bifulco, M., Laezza, C., Aloj, S. M., and Garbi, C., J. cell. Physiol. *155* (1993) 340.
29 Robinson, J. B., Inman, L., Sumegi, B., and Srere, P. A., J. biol. Chem. *262* (1987) 1786.
30 Sumegi, B., McCammon, M. T., Sherry, A. D., Keys, D. A., McAlister-Henn, L., and Srere, P. A., Biochemistry *31* (1992) 8720.
31 Erecinska, M., and Wilson, D. F., J. Memb. Biol. *70* (1982) 1.
32 Krebs, H. A., and Johnson, W. A., Enzymologia *4* (1937) 148.
33 Chinet, A., Experientia *46* (1990) 1194.
34 Brown, G. C., Biochem. J. *284* (1992) 1.
35 Kholodenko, B. N., Demin, O. V., and Westerhof, H. V., FEBS Lett. *320* (1993) 75.
36 Small, R., and Kacser, H., Eur. J. Biochem. *213* (1993) 75.
37 Förster, M. E. C., J. theor. Biol. *133* (1988) 1.
38 McLennan, P. A., McArdle, A., and Edwards, R. H. T., Biochem. J. *275* (1991) 477.
39 Chi, M. M.-Y., Hintz, C. S., McKee, D., Felder, S., Grant, N., Kaiser, K. K., and Lowry, O. H., Metabolism *36* (1987) 761.
40 Lilling, G., and Beitner, R., Biochem. Med. metab. Biol. *45* (1991) 319.
41 Glesby, M. J., Rosenmann, E., Nylen, E. G., and Wrogemann, K., Muscle & Nerve *11* (1988) 852.
42 Daut, J., and Elzinga, G., J. Physiol. *413* (1989) 379.
43 Phillips, S. K., Wiseman, R. W., Woledge, R. C., and Kushmerick, M. J., J. Physiol. *462* (1993) 135.
44 Chew, S. F., and Ip, Y. K., Comp. Biochem. Physiol. *104B* (1993) 681.
45 Wikman-Coffelt, J., Stefenelli, T., Wu, S. T., Parmley, W. W., and Jasmin, G., Circulation Res. *68* (1991) 45.
46 Adams, V., Griffin, L., Towbin, J., Gelb, B., Worley, K., and McCabe, E. R. B., Biochem. Med. metab. Biol. *45* (1991) 271.
47 Wallimann, T., Wyss, M., Brdiczka, D., Nicolay, K., and Eppenberger, H., Biochem. J. *281* (1992) 21.
48 Strickland, J. M., and Ellis, D. A., Nature *253* (1975) 464.
49 Cassens, R. G., Bocek, R. M., and Beatty, C. H., Am. J. Physiol. *217* (1969) 715.
50 Mayr, G. W., Eur. J. Biochem. *143* (1984) 513.
51 Wrogemann, K. J., and Pena, S. D., Lancet *1* (1976) 672.
52 Wiche, G., Briones, K., Koszka, C., Artlieb, U., and Krepler, R., EMBO J. *3* (1984) 991.
53 Niggli, V., and Burger, M. M., J. Membr. Biol. *100* (1987) 97.
54 Danowski, B. A., Imanaka-Yoshida, K., Sanger, J. M., and Sanger, J., J. Cell Biol. *118* (1992) 1411.
55 Straub, V., Bittner, R. E., Léger, J. L., and Voit, T., J. Cell Biol. *119* (1992) 1183.