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Skeletal Muscle of Patients with Duchenne's Muscular Dystrophy: Evidence of a Mitochondrial Proteolytic Factor Responsible for Calmitine Deficiency

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We studied the effect of mitochondrial extracts of skeletal muscle obtained from patients with Duchenne's muscular dystrophy (DMD) on calmitine of the mitochondrial matrix isolated from skeletal muscle of control mice. Our results in vitro clearly show that calmitine of the mitochondrial matrix of control muscle was degraded in the presence of mitochondrial extracts of muscle from DMD patients. The diseased muscle apparently contains an abnormal calmitine-specific proteolytic factor responsible for the calmitine deficiency previously observed in this tissue. As calmitine binds calcium and probably plays a role in regulating the balance of bound and free calcium within mitochondria, a calmitine deficiency could result in an overload of mitochondrial free calcium. Certain enzymes involved in ATP synthesis would be inhibited, resulting in the muscular degeneration characteristic of this myopathy. Our results suggest the cause of mitochondrial calcium overload and the events leading to muscular degeneration in this disease model. Abnormal protease activity could be the factor triggering all of these processes in the DMD patient. These findings suggest that it may now feasible to search for an efficient pharmacologic treatment for DMD.

The process of muscular degeneration in human myopathy is still unclear. Despite the discovery of the defective gene in DMD and the lack of its encoding protein dystrophin (1), the factor triggering muscle cell death in this disease has not been determined (2). Although our knowledge about dystrophin and related proteins has increased considerably in recent years, there is still no indication as to the precise role of this protein in the muscle cell (3) and thus of the effects due to its absence. The calcium overload observed in dystrophic muscle (4) could be partly responsible for the cellular degeneration observed in the muscle of myopathic man, but the abnormal process involved has not been elucidated. Our previous work has demonstrated that a calmitine deficiency exists in mitochondria of the skeletal muscle of the DMD patient (5). This mitochondrial protein, the only one to bind calcium in our experimental conditions, is located quite precisely in the mitochondrial matrix (6) and appears to be specific for fast skeletal muscle (7). The development of an experimental model enabled us to demonstrate that calmitine decrease after a single injection of chlorpromazine in the normal mouse was associated with an increase in mitochondrial free calcium and resulted in muscular degeneration (8). Moreover, precise determination of the specific localization of calmitine in the mitochondrial matrix and its calcium-binding property suggests that the essential role of this protein is to regulate the balance between bound and free calcium in the mitochondrial matrix (9). As it is known that the concentration of free calcium has an influence on various mitochondrial enzymes involved in ATP synthesis (10–13), the role of calmitine is probably primordial in the functioning of the muscle cell. Thus, a calmitine deficiency (probably leading to a free calcium overload and inhibition of certain enzymes) could cause muscular degeneration in some myopathies by blocking ATP synthesis. This calmitine deficiency observed in DMD (5) may be due to a lack of synthesis of this protein or to its continual degradation. We investigated whether a factor responsible for calmitine proteolysis and the resulting deficiency exists in the mitochondria of skeletal muscle of DMD patients. The work reported here clearly demonstrates the existence of a calmitine-specific proteolytic activity in muscle mitochondria of the DMD patients.

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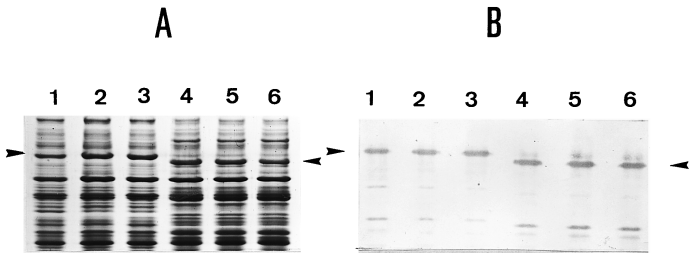

FIG. 1. Separation of mitochondrial proteins from control mouse gastrocnemius (lanes 1, 2, 3) and normal human quadriceps (lanes 4, 5, 6) muscle. Calmitine is indicated by arrow. A: Coomassie brilliant blue staining (protein sample on each lane 80 μg); B: Calmitine immunological visualization after blotting on a nitrocellulose sheet (protein sample on each lane 5 μg): mouse calmitine reacted with mouse anticalmitine (lane 1) and human anticalmitine (lanes 2, 3) antibodies; human calmitine reacted with human anticalmitine (lane 4) and mouse anticalmitine (lanes 5, 6) antibodies.

MATERIALS AND METHODS

Muscle biopsies of quadriceps muscle from DMD patients and control subjects were used for the preparation of mitochondrial extracts as previously described (5). After ether anesthesia, gastrocnemius muscle from adult male Swiss mice were removed and used for the preparation of subcellular fractions: mitochondria and mitochondrial matrix fractions were prepared as previously described (6). Fractions of reticulum sarcoplasmic were isolated as previously reported (9). Subcellular fraction proteins were assayed according to the method of Lowry (14).

In vitro experiments were performed as follows: the fractions of mitochondrial matrix and sarcoplasmic reticulum from control mice were incubated for 10 min at 37°C alone or in the presence of mitochondrial extracts from DMD patients or control subjects. After incubation, all samples were used for protein analysis by SDS polyacrylamide gel electrophoresis (15) in duplicate: one gel was stained with Coomassie brilliant blue and the other was used for Ca45 binding after protein blotting on nitrocellulose sheets (16). Calcium-45 protein binding was performed according to the technique of Maruyama (17) and measured by autoradiography. In some experiments, one gel was stained with Coomassie brilliant blue and the other was subjected to nitrocellulose blotting for immunological reaction of calmitine with anticalmitine antibody as previously described (16).

RESULTS AND DISCUSSION

Calmitine is present in the mitochondria of skeletal muscle in the normal subject and the control mouse (fig. 1A). There is a slight difference in molecular weight between human (58,000) and mouse (61,000) calmitine, but the calcium binding capacity is the same for both as previously observed (5). Human anticalmitine antibody recognizes mouse and human calmitine and the same

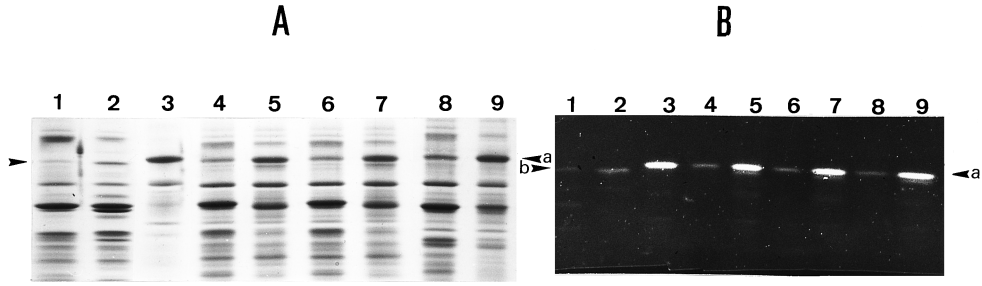

FIG. 2. Separation of mitochondrial matrix proteins isolated from mouse gastrocnemius muscles; matrix fractions were incubated alone (lane 3, control, 15 μg) or in the presence of quadriceps mitochondrial extracts from DMD patients or control subjects (20 μg). Calmitine (indicated by arrow, a: mouse calmitine; b: human calmitine) was degraded in the presence of quadriceps mitochondrial extracts from DMD patients (lanes 4, 6, 8); it was similar to control (lane 3) in the presence of quadriceps mitochondrial extracts from control subjects (lanes 5, 7, 9); lanes 1, 2: quadriceps mitochondrial extracts (40 μg) from DMD patient (lane 1) and control subject (lane 2). A: Coomassie brilliant blue staining. B: 45Ca binding proteins after autoradiography.

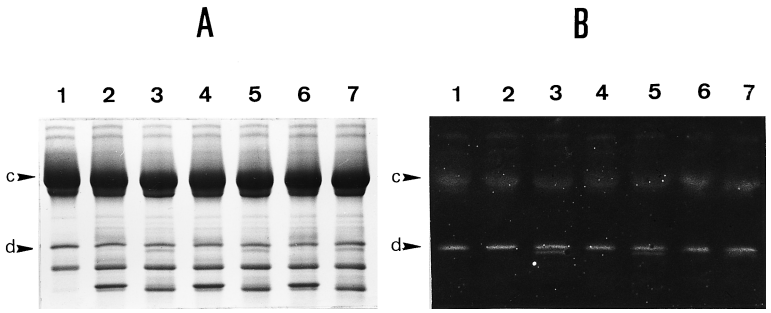

FIG. 3. Separation of sarcoplasmic reticulum proteins isolated from control mouse gastrocnemius muscles (protein sample: 50 μg) alone (lane 1, control) or in the presence of mitochondrial extracts from DMD patients (lanes 2, 4, 6) or control subjects (lanes 3, 5, 7). Ca-ATPase (c) and calsequestrin (d) (indicated by arrows) were not degraded in the presence of mitochondrial extracts from DMD patients or control subjects: they were identical to control (lane 1). (Protein sample on each lane: 50 μg). A: Coomassie brilliant blue staining. B: 45Ca binding proteins after autoradiography.

is true for mouse anticalmitine antibody (fig. 1B). The fact that this mouse and human protein does not migrate at the same level in gel electrophoresis (fig. 1) allows much more reliable interpretation of the cross-experimentation performed here: we tested the proteolytic effect of mitochondria from 3 DMD patients on calmitine from normal mice. Figure 2 shows that calmitine in the mitochondrial matrix of the control mouse was degraded by the presence of a mitochondrial extract from DMD patients. However, mitochondrial extracts from normal subjects caused no proteolysis of matrical calmitine. This proteolytic mitochondrial factor affecting calmitine and present in DMD patients had no proteolytic effect on proteins from sarcoplasmic reticulum, particularly CaATPase and calsequestrin (fig. 3). In all cases (fig. 2 B and 3B) the quantitative variations in bound Ca45 corresponded to those of calmitine observed after Coomassie brilliant blue staining.

We verified that the proteolysis of matrical calmitine in control muscle subjected to a mitochondrial extract of dystrophic muscle from DMD patients was not due simply to the presence of calcium in the extract. Fractions of mitochondrial matrix from control mouse muscle were incubated with increasing quantities of calcium, and the results (fig. 4) show that the specific protease of calmitine, isolated together with this protein in matrical fractions (9), was not apparently activated by an increase in calcium.

Our results clearly show the existence of a “proteolytic factor” in mitochondria of DMD patients, which specifically induces degradation of mitochondrial calmitine in vitro but has no proteolytic effect on CaATPase and calsequestrin in sarcoplasmic reticulum. This proteolytic factor could be a protease present both in normal and myopathic muscles, but inactive in the former and overly

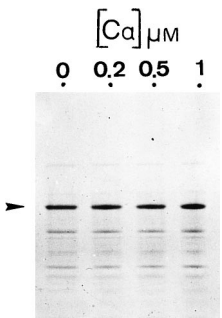

FIG. 4. Analysis of calmitine from matrix mitochondrial fractions of control mice gastrocnemius muscle: matrix fractions were incubated for 20 min at 37°C with calcium increasing concentrations from 0 to 1 μM (0 to 10 nM/mg protein). Protein sample on each lane: 10 μg. There was no degradation of calmitine in the presence of calcium.

active in the latter. In fact, our previous results (9) showed that a protease specific for calmitine exists in fractions of mitochondrial matrix isolated from control skeletal muscle. In addition, a protease specific for CaATPase and calsequestrin exists in fractions of sarcoplasmic reticulum isolated from control skeletal muscle. These proteases are inactive in normal conditions but are activated by addition of chlorpromazine, thereby causing protein degradation (9). This calmitine protease would appear to be activated in the presence of mitochondria of DMD muscle. There was no effect on CaATPase and calsequestrin proteases of sarcoplasmic reticulum. The fact that calmitine proteolysis in normal muscle can be induced by the addition of a mitochondrial extract from diseased muscle suggests the following hypothesis: mitochondria of diseased muscle may possess a normal protease, identical to that of control muscle, which is activated by a factor X exclusively present in diseased muscle. The addition of diseased mitochondria to the mitochondrial matrix of normal muscle could supply factor X, thereby inducing activation of the normally inactive protease and causing calmitine degradation. It is tempting to suppose that the factor X is calcium. Our results show that a calcium increase in the matrical fraction of normal muscle had no effect on calmitine (fig. 4). Thus, the activation factor may not be calcium but another agent present only in the mitochondria of diseased muscle. The fact that calcium does not appear to be responsible for the activation of this protease in diseased muscle suggests the following mechanism in DMD patients: the abnormally active protease specific for calmitine and present in diseased muscle induces continual degradation of this protein as its synthesis occurs, thereby accounting for the marked calmitine deficiency in these patients (5). As calmitine possesses a calcium-binding property and is specifically localized in the mitochondrial matrix (6), it is very likely to play an important role in regulating the balance between bound and free calcium within mitochondria. As an experimental model has shown that calmitine reduction leads to a rise in mitochondrial free calcium (8), calmitine deficiency may be responsible for the free calcium overload in DMD muscle mitochondria: calmitine concentration would no longer be sufficient to bind calcium, leading to an excess of free calcium. Since the activity of the various mitochondrial enzymes involved in ATP synthesis depends on the concentration of free calcium, an excess of this metal could inhibit these enzymes, causing a blockage of ATP synthesis and resulting in cell death (18).

The results reported here suggest that calmitine deficiency could be due to proteolysis induced by a protease abnormally active in the muscles of DMD patients. This could be the factor triggering the chain of mitochondrial processes leading to the muscular degeneration characteristic of this disease. The inhibition of this abnormal proteolytic activity could constitute a new approach for efficient pharmacological treatment of certain forms of human myopathy.

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REFERENCES

1. Hoffman, E. P., Brown, R. H. Jr., and Kunkel, L. M. (1987) *Cell* **51,** 919–928.
2. Tidball, J. G., Albrecht, D. E., and Lokensgard, B. E., *et al.* (1995) *J. Cell. Sci.* **108,** 2197–2204.
3. Tinsley, J. M., Blake, D. J., and Zuellig, R. A., *et al.* (1994) *Proc. Natl. Acad. Sci. USA* **91,** 8307–8313.
4. Morandi, L., Mora, M., and Gussoni, E. (1990) *Ann. Neurol.* **28,** 674–679.
5. Lucas-Heron, B., Mussini, J. M., and Ollivier, B. (1989) *J. Neurol. Sci.* **90,** 299–306.
6. Bataille, N., Schmitt, N., Aumercier-Maes, P., Ollivier, B., and Lucas-Heron, B., *et al.* (1994) *Biophys. Res. Commun.* **203,** 1477–1482.
7. Lucas-Heron, B., Schmitt, N., and Ollivier, B. (1990) *Neurosci. Lett.* **115,** 103–107.
8. Schmitt, N., Lucas-Heron, B., Ollivier, B., and Mussini, J. M. (1991) *J. Neurol. Sci.* **105,** 44–48.
9. Lucas-Heron, B., Le Ray, B., and Schmitt, N. (1995) *FEBS Lett.* **374,** 309–311.
10. Denton, R. M., Randle, P. J., and Martin, B. R. (1972) *Biochem. J.* **128,** 161–163.

11. Denton, R. M., Richards, D. A., and Chin, J. G. (1978) *Biochem. J.* **176,** 894–906.
12. Mc Cormack, J. G., and Denton, R. M. (1979) *Biochem. J.* **180,** 533–544.
13. Harris, D. A., and Das, A. M. (1991) *Biochem. J.* **280,** 561–573.
14. Lowry, O. H., Rosebrough, N. J., and Farr, A. L., *et al.* (1951) *J. Biol. Chem.* **193,** 265–275.
15. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
16. Lucas-Heron, B., Schmitt, N., and Ollivier, B. (1990) *J. Neurol. Sci.* **95,** 327–334.
17. Maruyama, K., Mikawa, T., and Ebashi, S. (1984) *J. Biochem.* **95,** 511–519.
18. Wrogeman, K., and Pena, S. D. J. (1976) *Lancet* **1,** 672–673.