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Altered threshold of the mitochondrial permeability transition pore in Ullrich congenital muscular dystrophy

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ABSTRACT

We have studied the effects of rotenone in myoblasts from healthy donors and from patients with Ullrich congenital muscular dystrophy (UCMD), a severe muscle disease due to mutations in the genes encoding the extracellular matrix protein collagen VI. Addition of rotenone to normal myoblasts caused a very limited mitochondrial depolarization because the membrane potential was maintained by the F1FO synthase, as indicated by full depolarization following the subsequent addition of oligomycin. In UCMD myoblasts rotenone instead caused complete mitochondrial depolarization, which was followed by faster ATP depletion than in healthy myoblasts. Mitochondrial depolarization could be prevented by treatment with cyclosporin A and intracellular Ca^{2+} chelators, while it was worsened by depleting Ca^{2+} stores with thapsigargin. Thus, in UCMD myoblasts rotenone-induced depolarization is due to opening of the permeability transition pore rather than to inhibition of electron flux as such. These findings indicate that in UCMD myoblasts the threshold for pore opening is very close to the resting membrane potential, so that even a small depolarization causes permeability transition pore opening and precipitates ATP depletion.

1. Introduction

Ullrich congenital muscular dystrophy (UCMD) is a severe muscle disease due to mutations in the genes encoding collagen VI, an extracellular matrix protein forming a microfibrillar network that is particularly prominent in the endomysium of skeletal muscle [1,2]. Myofibers from the *Col6a1*$^{-/-}$ murine model of the disease [3,4] and myoblasts from UCMD patients [5] display a latent mitochondrial dysfunction linked to opening of the permeability transition pore (PTP) [4,5], an inner membrane high-conductance channel whose inappropriate openings cause functional and ultrastructural alterations of mitochondria, and spontaneous apoptosis. These alterations could be normalized by treatment with cyclosporin (Cs) A [4,5], a widely used immunosuppressant that desensitizes the PTP independent of calcineurin inhibition [6].

Mitochondrial dysfunction is latent in the sense that cultured *flexor digitorum brevis* fibers from *Col6a1*$^{-/-}$ mice and myoblasts from UCMD patients display a normal mitochondrial membrane potential; yet, and at variance from fibers of wild-type mice and from myoblasts of healthy donors, they undergo mitochondrial depolarization upon the addition of the F1FO ATP synthase inhibitor oligomycin [4,5]. We have interpreted these results to mean that mitochondria in cells of organisms lacking collagen VI are not respiring normally, possibly because of gradual depletion of pyridine nucleotides following brief PTP openings [7,8]; and appear to use ATP produced by glycolysis to maintain their membrane potential [4,5]. In this manuscript we have further explored mitochondrial bioenergetics in collagen VI myopathies by studying the response of UCMD myoblasts to rotenone, the specific inhibitor of complex I. Unexpectedly we observed that, like oligomycin, rotenone caused a profound depolarization of UCMD myoblasts that could be inhibited by CsA and by intracellular Ca^{2+} chelators. These findings shed new light on the basis for mitochondrial dysfunction in UCMD, and suggest that (i) in collagen VI diseases the threshold voltage for PTP opening is very close to the resting potential, a condition that substantially increases the probability that the pore may open after small depolarizations; and (ii) the depolarizing effect of oligomycin may be indirect and secondary to ATP depletion and Ca^{2+} deregulation, which would further shift the PTP voltage threshold toward the resting potential causing pore opening.

2. Materials and methods

The preparation of myoblast cultures from muscle biopsies of healthy donors and UCMD patients and the determination of the mitochondrial membrane potential were carried out exactly as described previously [5]. ATP levels were measured using the Luminescence ATP Detection Assay System (ATPlite, PerkinElmer). Myoblasts were seeded onto 96-well microplate and grown for two days in Dulbecco's modified Eagle's medium supplemented with 20% fetal calf serum. Samples were lysed according to the

Abbreviations: BAPTA-AM, 1,2-bis(*o*-aminophenoxy)-ethane-N,N,N′,N′-tetraacetc acid tetraacetoxymethyl ester; Cs, Cyclosporin; EGTA, ethylene glycol-bis(2-aminoethyl)-N,N,N′,N′-tetraacetic acid; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenyl hydrazone; PTP, permeability transition pore; SR, sarco-endoplasmic reticulum; TMRM, tetramethylrhodamine methyl ester; UCMD, Ullrich congenital muscular dystrophy

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manufacturer's instructions and luminescence measured with a fluorimeter plate reader (Fluoroscan, PerkinElmer). Results obtained with healthy donor and UCMD patient cells subjected to different treatments were analyzed with 2-way ANOVA (significant values with $P<0.05$). In the cases where we obtained statistically significant differences we applied *post hoc* ANOVA with Bonferroni correction to compare the means between independent groups (significant values with $P<0.05$).

3. Results

We monitored the membrane potential of mitochondria *in situ* by measuring their accumulation of tetramethylrhodamine methyl ester (TMRM). Under our loading conditions mitochondrial depolarization corresponds to a decrease of mitochondrial fluorescence, since the accumulated probe is still below the quenching threshold [9]. It should be noted that cells were also treated with CsH, which does not affect the PTP but inhibits the multidrug resistance pump and therefore normalizes cytosolic loading with TMRM, which is a substrate of the pump and could therefore be extruded at rates that vary widely in different cell types [10]. Addition of the complex I inhibitor rotenone to healthy myoblasts caused a slight decrease of the mitochondrial membrane potential, which remained then stable until the protonophore carbonylcyanide-*p*-trifluoromethoxyphenyl hydrazone (FCCP) was added (Fig. 1, open circles). This finding suggests that the initial depolarization triggered the reversal of the F1FO ATP synthase, which was then working as a proton pump to maintain the mitochondrial membrane potential at the expense of glycolytic ATP. Indeed, addition of the ATP synthase inhibitor oligomycin to rotenone-treated myoblasts promptly induced mitochondrial depolarization (Fig. 1, closed circles). We then analyzed the response to rotenone of myoblasts from one UCMD patient bearing a *COL6A3* homozygous nonsense Arg465Stop mutation (further details can be found in a previous publication [5]). At striking variance from the cells of the healthy donor, UCMD myoblasts depolarized rapidly and completely upon the addition of rotenone alone (Fig. 1, open squares). A similar response was observed in myoblasts of UCMD patients with different genetic lesions of the collagen VI genes (results not shown).

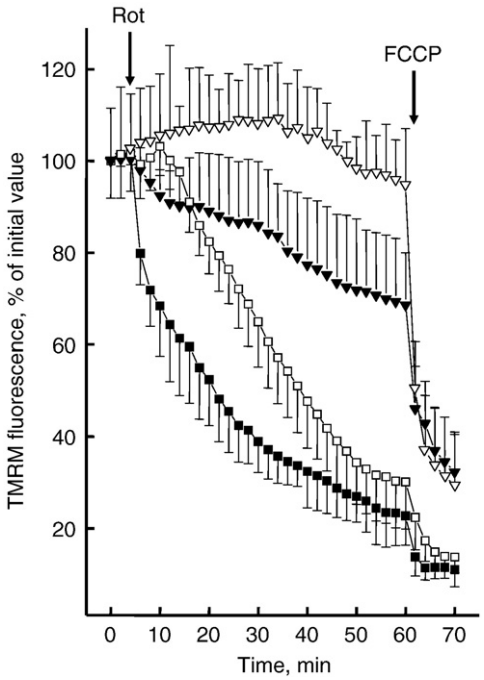

Fig. 2. Effects of Ca^{2+} chelators and CsA on mitochondrial TMRM fluorescence changes induced by rotenone in UCMD myoblasts. UCMD myoblasts were loaded with TMRM and studied as described [5]. When indicated by arrows 4 μM rotenone (Rot) and 4 μM FCCP were added in the absence of further treatments (closed squares) or after treatment for 30 min with 1.6 μM CsA (open squares), 5 μM BAPTA-AM and 1 mM EGTA (closed triangles) or 1.6 μM CsA, 5 μM BAPTA-AM and 1 mM EGTA (open triangles).

We investigated the mechanistic basis for the rotenone effect in UCMD myoblasts. Treatment with CsA delayed the onset of rotenone-induced depolarization but was unable to prevent it (Fig. 2, compare open with closed squares). Mitochondrial depolarization was substantially

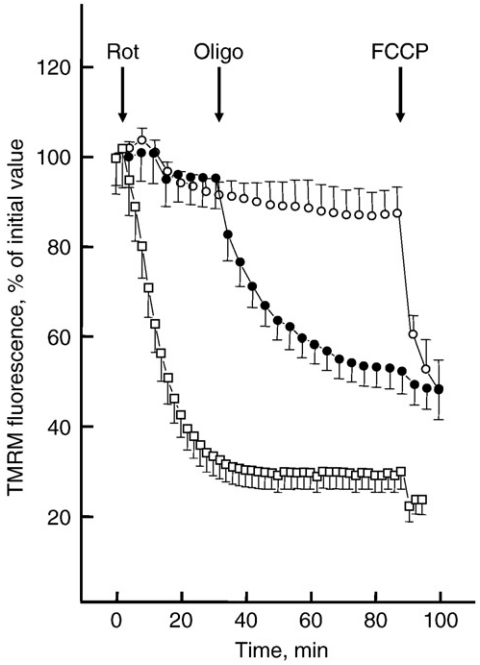

Fig. 1. Changes of mitochondrial TMRM fluorescence induced by rotenone in healthy and UCMD myoblasts. Myoblasts from one healthy donor (circles) and from one UCMD patient characterized in a previous study [5] (squares) were loaded with TMRM and studied as described [5]. When indicated by arrows 4 μM rotenone (Rot) was added (all traces) followed by 6 μM oligomycin (Oligo) (closed circles only) and 4 μM FCCP (all traces).

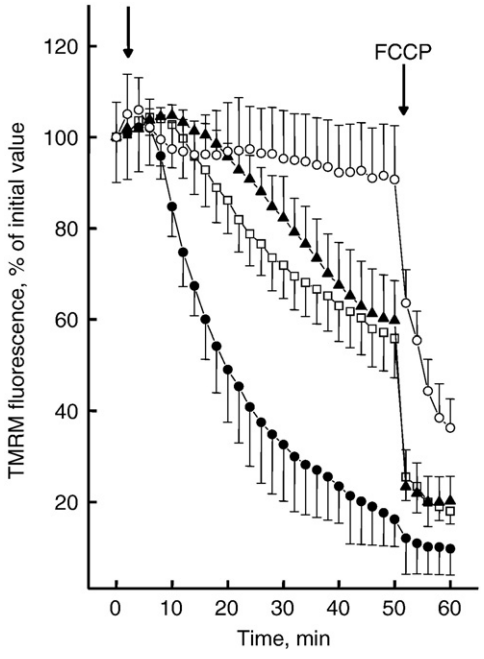

Fig. 3. Changes of mitochondrial TMRM fluorescence induced by thapsigargin and oligomycin in UCMD myoblasts. Myoblasts were loaded with TMRM and studied as described [5]. When indicated by the first arrow the following additions were made: thapsigargin (closed triangles); oligomycin (open squares); thapsigargin and oligomycin (closed circles); thapsigargin and oligomycin in cells pretreated with 1.6 μM CsA for 30 min (open circles). The concentrations of thapsigargin and oligomycin were 10 μM and 6 μM, respectively. Where indicated 4 μM FCCP was added.