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

Alessia Angelin [a], Paolo Bonaldo [b], Paolo Bernardi [a,*]

[a] Department of Biomedical Sciences and CNR Institute of Neuroscience, University of Padova, Italy
[b] Department of Histology, Microbiology and Medical Biotechnologies, University of Padova, Italy

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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.

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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

* Corresponding author. Department of Biomedical Sciences, University of Padova, Viale Giuseppe Colombo 3, I-35121 Padova, Italy. Fax: +39 049 827 6361.
E-mail address: bernardi@bio.unipd.it (P. Bernardi).

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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).

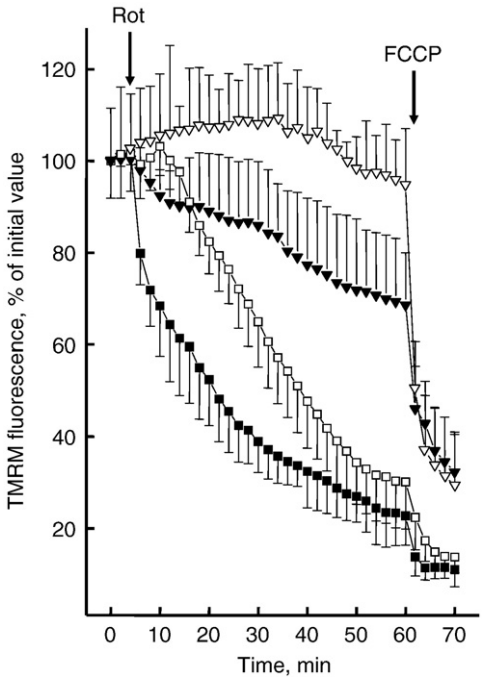

Fig. 2. Effects of Ca^{2+} chelators and CsA on mitochondrial TMRM fluorescence changes induced by rotenone in UCMD myoblasts. UCMD myoblasts were loaded with TMRM and studied as described [5]. When indicated by arrows 4 μM rotenone (Rot) and 4 μM FCCP were added in the absence of further treatments (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

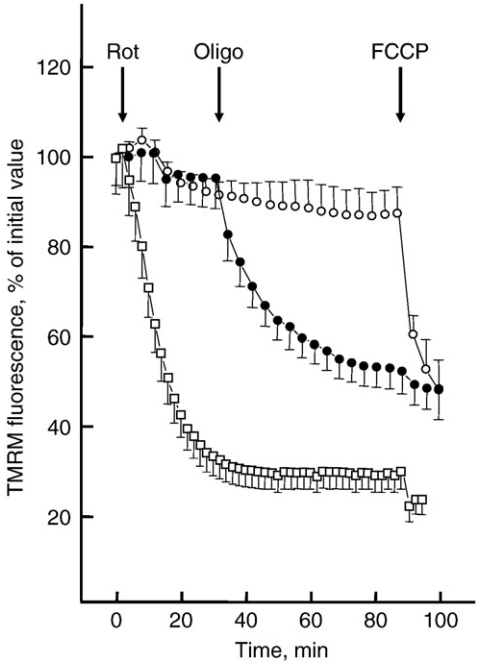

Fig. 1. Changes of mitochondrial TMRM fluorescence induced by rotenone in healthy and UCMD myoblasts. Myoblasts from one healthy donor (circles) and from one UCMD patient characterized in a previous study [5] (squares) were loaded with TMRM and studied as described [5]. When indicated by arrows 4 μM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

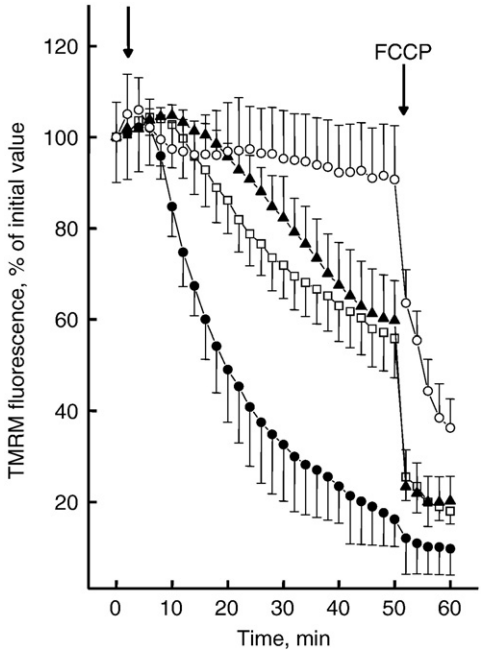

Fig. 3. Changes of mitochondrial TMRM fluorescence induced by thapsigargin and oligomycin in UCMD myoblasts. Myoblasts were loaded with TMRM and studied as described [5]. When indicated by the first arrow the following additions were made: thapsigargin (closed triangles); oligomycin (open squares); thapsigargin 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.

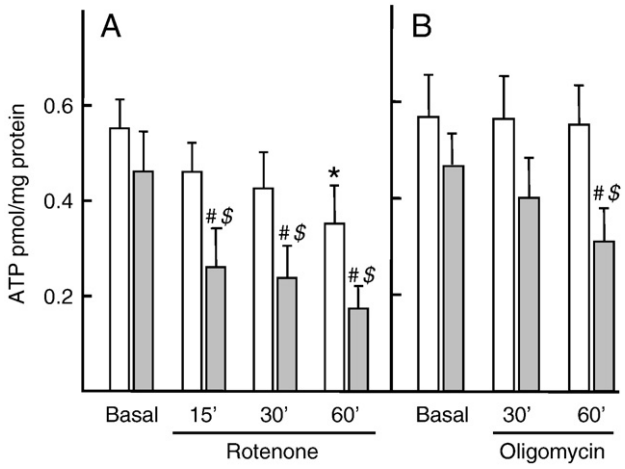

Fig. 4. Effects of rotenone and oligomycin on ATP levels in healthy and UCMD myoblasts. ATP concentration was measured in myoblasts from healthy donor (open columns) and UCMD patient (closed columns) as described in Materials and Methods in the absence of additions (Basal) or after treatment with 4 μM rotenone (panel A) or 6 μM oligomycin (panel B) for the indicated times. The results shown are representative of at least five repeats per condition ± SD. * $P<0.05$ versus healthy donor basal condition; # $P<0.05$ versus UCMD patient basal condition and $ $P<0.05$ versus healthy donor.

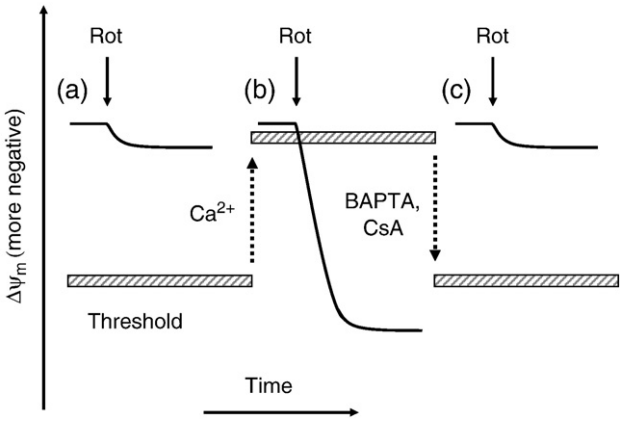

Fig. 5. Shift of PTP voltage threshold in UCMD. The scheme illustrates how a shift of the threshold voltage for PTP opening by Ca^{2+} (hatched bar) could cause PTP opening by rotenone, an event that is reversed by BAPTA and CsA. For further explanation see text.

slowed down by treatment with the intracellular Ca^{2+} chelator 1,2-bis(*o*-aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid tetraacetoxymethyl ester (BAPTA-AM) plus ethylene glycol-bis(2-aminoethyl)-N,N,N′,N′-tetraacetic acid (EGTA) (Fig. 2, closed triangles), an effect that could also be obtained by adding BAPTA-AM but not EGTA alone (omitted for clarity). Strikingly, the combination of CsA, BAPTA-AM and EGTA completely prevented rotenone-induced depolarization (Fig. 2, open triangles), while a collapse of the mitochondrial membrane potential readily followed the addition of FCCP (Fig. 2, all traces). These findings indicate that the depolarizing response of UCMD myoblasts to rotenone is due to an alteration of intracellular Ca^{2+} homeostasis affecting the PTP rather than to inhibition of complex I as such.

In the light of these observations, we investigated whether discharge of Ca^{2+} from the sarco-endoplasmic reticulum (SR) with thapsigargin could affect the PTP. Strikingly, addition of thapsigargin caused mitochondrial depolarization (Fig. 3, triangles) which could be inhibited by CsA (results not shown). The depolarizing effect of rotenone in UCMD myoblasts is so fast (see Fig. 1) that it is difficult to assess the possible additional effect of thapsigargin. We therefore studied the response to oligomycin, which also initiates mitochondrial depolarization in UCMD myoblasts, often after a lag phase (Fig. 3, open squares and [5]). The addition of thapsigargin plus oligomycin caused a strong potentiation of mitochondrial depolarization (Fig. 3, closed circles), which remained fully sensitive to inhibition by CsA (Fig. 3, open circles).

We also studied the changes of cellular ATP levels induced by rotenone and oligomycin. Rotenone caused a time-dependent decrease of total cellular ATP, and the rate of decrease was faster in UCMD myoblasts (Fig. 4A). Normal myoblasts were rather resistant to ATP depletion by oligomycin, whereas UCMD myoblasts underwent a decrease of about 50% of their ATP content in the 1-h time frame of these experiments (Fig. 4B).

4. Discussion

This study sheds new light on the basis for mitochondrial dysfunction in UCMD, a muscular dystrophy caused by collagen VI deficiency. We have demonstrated that the depolarizing response to rotenone is not due to inhibition of respiration *per se*, but rather to opening of the PTP. This finding demands a reassessment of our working hypotheses on respiratory dysfunction in myoblasts and fibers from *Col6a1*$^{-/-}$ mice [4] and in myoblasts from UCMD patients [5].

4.1. Mechanism of rotenone-induced depolarization in UCMD myoblasts

The scheme of Fig. 5 depicts our model to explain the effects of rotenone in normal and UCMD myoblasts. In healthy cells, addition of rotenone causes a decrease of mitochondrial membrane potential that is readily compensated by reversal of the ATP synthase, thus preventing substantial depolarization (Fig. 5a). The threshold voltage for PTP opening is not reached and the inner membrane permeability remains low, allowing the maintenance of the membrane potential at the expense of ATP hydrolysis. In UCMD myoblasts, the PTP threshold is instead very close to the resting membrane potential (as a result of increased matrix Ca^{2+} levels and/or of PTP sensitization to other factors), a condition that causes PTP opening even for small depolarizations (Fig. 5b). Because of the increased inner membrane permeability repolarization cannot occur despite ATP hydrolysis, which indeed proceeds at a faster rate (Fig. 4B). PTP sensitization can be removed by Ca^{2+} chelation with BAPTA-AM or by inhibition of cyclophilin D with CsA, which will move the threshold away from the resting potential and

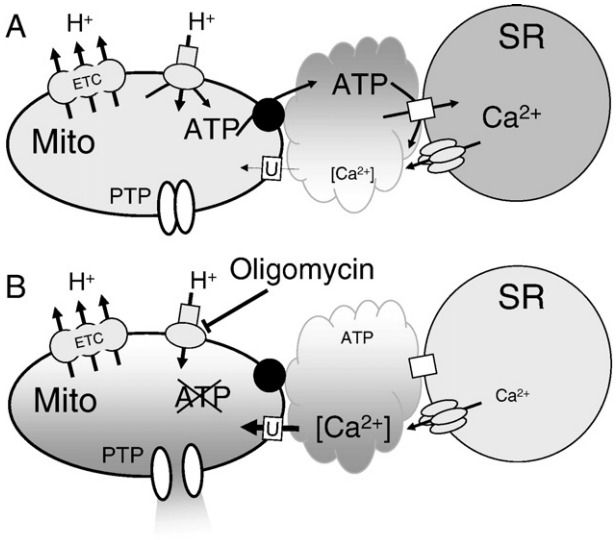

Fig. 6. Mitochondria-SR interactions in regulation of ATP production and Ca^{2+} homeostasis. Effect of oligomycin. Panel A, under normal conditions the proton gradient created across the inner membrane of mitochondria (Mito) by the electron transfer chain (ETC) is used to synthesize ATP, which is exported and channelled to the SR where it fuels the Ca^{2+} pumps. Ca^{2+} release at the mouth of the release channels is relayed to the matrix through the Ca^{2+} uniporter (U) to regulate ATP synthesis. Panel B, inhibition of the ATP synthase by oligomycin decreases the ATP available to the Ca^{2+} pumps and causes a shift of Ca^{2+} from the SR to mitochondria, eventually resulting in PTP opening that is particularly likely to occur in UCMD. For further explanation see text.

restore the normal response to rotenone (Fig. 5c). This explanation is in keeping with all the experimental results of this manuscript, including the partial sensitivity of rotenone-dependent mitochondrial depolarization to CsA in UCMD myoblasts. Indeed, as the mitochondrial Ca^{2+} load is raised the PTP opens irrespective of cyclophilin D inhibition, an effect that is also observed in mitochondria from mice where expression of cyclophilin D has been selectively ablated [11–14].

4.2. Mechanism of oligomycin-induced depolarization in UCMD myoblasts

If mitochondria in UCMD myoblasts are respiring (as indicated by their depolarizing response to rotenone) why do they also undergo depolarization upon the addition of the F1FO ATP synthase inhibitor oligomycin (Fig. 3 and [5]), an effect that is also observed in muscle fibers from *Col61a*$^{-/-}$ mice [4]? As noted earlier, the depolarizing effect of oligomycin indicates that mitochondria are using glycolytic ATP to maintain their membrane potential [4,5]. The data of the present manuscript suggest that oligomycin may affect pore opening through a shift of the voltage threshold due the Ca^{2+} overload, an event where dysfunction of the SR could be critical. As depicted in the scheme of Fig. 6A for normal myoblasts, the SR plays a key role in Ca^{2+} homeostasis in close collaboration with the mitochondria, which provide the ATP required for SR Ca^{2+} uptake, an event that becomes critical for muscle relaxation in excitation-contraction coupling of differentiated skeletal muscle fibers [15,16]. The close interactions between the two organelles also allow transfer of Ca^{2+} to the mitochondria from "hot spots" near the mouth of the Ca^{2+} release channels without major changes of the average cytosolic $[Ca^{2+}]$ [17,18]. Addition of oligomycin causes inhibition of mitochondrial ATP synthesis, which is followed by a rapid drop of total cellular ATP only in the UCMD myoblasts (Fig. 4B). This will cause decreased Ca^{2+} uptake by the SR (Fig. 6B) and decreased Ca^{2+} extrusion by the plasma membrane Ca^{2+} pumps (not illustrated in the scheme for clarity), resulting in an increase of the free Ca^{2+} concentrations near the mitochondria, with progressive transfer of Ca^{2+} to the matrix, where it would cause a gradual shift of the PTP voltage threshold and pore opening as soon as the threshold reaches the resting membrane potential (Fig. 6B). It is noteworthy that oligomycin-induced mitochondrial depolarization is often preceded by a lag phase, which may well represent the time required for the shift of the threshold; that it is prevented by intracellular Ca^{2+} chelators; and that it spreads rather slowly, suggesting that different mitochondria have a different threshold for pore opening, the most resistant being recruited by the spreading wave of Ca^{2+} released by the mitochondria that open the PTP first (see [19] for a review).

4.3. Mitochondrial dysfunction in UCMD. A working hypothesis

In summary, we believe that in UCMD the threshold for PTP opening is dangerously close to the resting potential because of altered Ca^{2+} homeostasis and of additional factors that are under active investigation. These might involve overproduction or decreased scavenging of reactive oxygen species [20], which affect the PTP through thiol oxidation [21,22]. This situation exposes skeletal muscle fibers, which depend on collagen VI for survival, to the hazard of increased frequency of pore opening during physiological depolarizations, such as those due to an increased energy demand by the contractile apparatus. Increased PTP opening could slowly decrease the matrix content of pyridine nucleotides and hence the maximal respiratory capacity [7,8], eventually switching the F1FO ATP synthase into an ATP-consuming device. Worsening of SR performance would then cause overt Ca^{2+} deregulation, stabilize the PTP open state and cause hypercontracture and fiber cell death. Studies are under way in both the animal models and in the human cell cultures to directly test these hypotheses.

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