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A PATHOGENIC ROLE FOR MITOCHONDRIA IN COLLAGEN VI MUSCULAR DYSTROPHIES

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ABSTRACT

Mitochondria are essential organelles not only in the life of the cell, but also during death. Indeed, they play a key role in the conservation of energy (in the form of ATP) and in the signaling pathways that lead cells to die. Cell death is regulated by many mechanisms in a wide variety of conditions. One of the most important events in this pathway is the permeability transition, a sudden increase of the inner mitochondrial membrane permeability to solutes with molecular mass below 1500 Da. The entity responsible for this phenomenon is the permeability transition pore (PTP), a high conductance inner membrane channel. Opening of this pore causes several effects such as depolarization, osmotic swelling, cristae remodeling that may result in the rupture of the outer membrane, and eventually in the release of apoptogenic proteins, usually locked up in the mitochondria. The PTP is widely believed to be involved in the pathogenesis of several diseases, and a striking example is represented by CollagenVI-related disorders.

Ullrich congenital muscular dystrophy (UCMD) and Bethlem myopathy (BM) are inherited muscle diseases caused by mutations of genes encoding the extracellular matrix protein Collagen (Col) VI. Studies on the mouse model of these disorders (*Col6a1*^{-/-}) and on patients have shown an increased rate of spontaneous apoptosis, ultrastructural alterations in mitochondria and sarcoplasmic reticulum, and a latent mitochondrial dysfunction with abnormal opening of the PTP in skeletal muscle. Moreover, treatment with Cyclosporin (Cs) A, an immunosuppressive drug used against transplant rejection that desensitizes the PTP by binding to Cyclophilin (CyP)-D, rescues myofibers alterations both in *Col6a1*^{-/-} mice and in UCMD patients. It should be noted, however, that CsA binds to, and inhibits, all members of the family of CyPs (16 different proteins), which play important roles in pathophysiology. Thus, despite the therapeutic efficacy of this drug in ColVI myopathies, it is impossible to sort the consequences of CyP-D inhibition from those caused by inhibition of other CyPs. Furthermore, CsA also inhibits calcineurin, which has been shown to affect skeletal muscle physiology and mitochondrial shape through the involvement of the pro-fission dynamin related protein-1 (Drp-1).

The first aim of the work presented in this Thesis was to clarify whether the beneficial effect of CsA in ColVI dystrophies depends uniquely on PTP desensitization through its inhibition of CyP-D, or on the action of this drug on additional targets. We have crossed *Col6a1*^{-/-} and *Ppif*^{-/-} mice (*Ppif* is the gene coding for CyP-D) to obtain a *Col6a1*^{-/-}*Ppif*^{-/-} strain where both genes have been inactivated; and we have studied the phenotype of these animals to determine whether the absence of CyP-D caused a rescue from the disease phenotype. *Col6a1*^{-/-}*Ppif*^{-/-} mice show negligible myofiber degeneration, rescue from mitochondrial dysfunction and ultrastructural defects, and normalized incidence of apoptosis. These findings (i) demonstrate that lack of CyP-D is equivalent to its inhibition with CsA at curing the mouse dystrophic phenotype; (ii) establish a cause-effect relationship between CyP-D-dependent PTP regulation and pathogenesis of the ColVI-

related disorders; and (iii) validate CyP-D and the PTP as pharmacological targets for the therapy of human ColVI myopathies.

The second aim of the work was to establish whether the remarkable therapeutic effects of CsA are also observed with the Cs derivative Debio 025 (D-MethylAlanine³-EthylValine⁴-cyclosporin), which maintains the ability to bind to, and inhibit, CyPs but does not inhibit calcineurin. We therefore studied NF-AT translocation, T cell activation, propensity to open of the PTP in mitochondria and skeletal muscle fibers, muscle ultrastructure and apoptotic rates in *Col6a1*^{-/-} mice before and after treatment with Debio 025. The key results we have obtained are: (i) Debio 025 does not inhibit calcineurin, yet it desensitizes the PTP *in vivo*; (ii) treatment with Debio 025 prevents mitochondrial dysfunction and normalizes the apoptotic rates and ultrastructural lesions of myopathic *Col6a1*^{-/-} mice. Thus, desensitization of the PTP can be achieved by selective inhibition of CyP-D without inhibition of calcineurin, resulting in an effective therapy of myopathic mice. These findings prove that ColVI dystrophies can be treated with Debio 025; and represent an essential step toward a therapy of UCMD and BM because Debio 025 does not expose patients to the potential harmful effects of immunosuppression.

A third question is whether the anomalous depolarizing response to oligomycin, which we have observed in cells from all patients affected by ColVI-related disorders, is a distinctive feature of these diseases, a point that has been questioned in a recent publication. We have studied the mitochondrial response in primary cell cultures derived from muscle biopsies of UCMD and BM patients to different stimuli according to the number of cell passages. Our results indicate that mitochondrial dysfunction is lost after 7 passages in culture, a time when cells also acquire a remarkable resistance to several apoptogenic treatments. These data are compatible with the selection of apoptosis-resistant cells that are presumably present in low numbers in the starting population. Thus, mitochondrial dysfunction, and in general the presence of a pathological phenotype, should be assessed early, and the number of passages is a critical factor to consider when results from different patients, and possibly different pathologies, are compared.

SOMMARIO

I mitocondri sono organelli essenziali nella vita e nella morte delle cellule. Giocano, infatti, un ruolo chiave sia nella conservazione dell'energia (sottoforma di ATP), che nelle vie di segnale che causano la morte della cellula. La morte cellulare è un processo regolato da molteplici meccanismi ed eventi, tra cui uno dei più importanti è la transizione di permeabilità, ovvero l'aumento improvviso della permeabilità della membrana mitocondriale interna a soluti con massa inferiore a circa 1500 Da. Questo fenomeno è causato dall'apertura di un canale ad alta conduttanza presente nella membrana interna, il poro di transizione di permeabilità (PTP). L'apertura del PTP provoca, nei mitocondri, una serie di effetti a catena, come la depolarizzazione, il rigonfiamento osmotico e il rimodellamento delle cristae, che termina con la rottura della membrana mitocondriale esterna e il conseguente rilascio di proteine pro-apoptotiche nel citoplasma. Il PTP, negli ultimi anni, ha acquisito un ruolo sempre più importante nella patogenesi di diverse malattie, tra cui un esempio interessante è rappresentato dalle distrofie muscolari da *deficit* di collagene VI.

La distrofia muscolare congenita di Ullrich (UCMD) e la miopatia di Bethlem (BM) sono due patologie ereditarie causate da mutazioni nei geni che codificano per il collagene VI, un'importante proteina della matrice extracellulare. Studi condotti sul modello murino per queste patologie (il topo *Col6a1*^{-/-}) e su pazienti affetti da UCMD, hanno evidenziato, a livello dei muscoli scheletrici, un aumento dell'apoptosi spontanea, di alterazioni ultrastrutturali dei mitocondri e del reticolo sarcoplasmatico e la presenza di una disfunzione mitocondriale latente con anormale apertura del PTP. Inoltre, è stato dimostrato che il trattamento con ciclosporina (Cs) A, un farmaco immunosoppressore usato nella terapia antirigetto per i trapianti che desensibilizza il PTP attraverso il legame con la ciclofilina (CiP)-D, causa il recupero delle alterazioni muscolari sia nei topi *Col6a1*^{-/-} che nei pazienti. E', però, importante sottolineare che la CsA lega e inibisce non solo la CiP-D, ma anche gli altri membri della famiglia delle ciclofiline (16 proteine diverse), i quali sono coinvolti nella patogenesi di diverse malattie. Non è, perciò, possibile separare gli effetti causati dall'inibizione della CiP-D da quelli sulle altre ciclofiline. Inoltre, la CsA inibisce anche la calcineurina, una fosfatasi coinvolta nei meccanismi che influenzano la crescita e lo sviluppo del muscolo scheletrico e la morfologia dei mitocondri, quest'ultima attraverso l'azione su una proteina (*dynamamin related protein-1*) importante per la fissione mitocondriale.

Il primo obiettivo del lavoro presentato in questa tesi è stato quello di chiarire se gli effetti terapeutici della CsA, riscontrati nelle distrofie muscolari da deficit di collagene VI, dipendessero unicamente dalla desensibilizzazione del PTP attraverso il legame con la CiP-D, oppure se fossero dovuti all'azione di questo farmaco su altri bersagli cellulari. A questo proposito, topi *Col6a1*^{-/-} sono stati incrociati con topi *Ppif*^{-/-} (*Ppif* è il gene che codifica per la CiP-D) in modo da ottenere topi *Col6a1*^{-/-}*Ppif*^{-/-} con entrambi i geni inattivati. I topi *Col6a1*^{-/-}*Ppif*^{-/-} sono stati studiati per valutare se l'assenza di CiP-D causasse il

recupero del fenotipo patologico. Questi animali non presentavano degenerazione a livello delle fibre muscolari, né disfunzioni mitocondriali o difetti ultrastrutturali, e i livelli di apoptosi erano normali. Si è giunti, perciò, ad alcune conclusioni: (i) la mancanza di CiP-D equivale alla sua inibizione da parte della CsA nella cura del fenotipo distrofico murino, (ii) esiste una relazione causa-effetto tra la regolazione del PTP da parte della CiP-D e la patogenesi delle distrofie muscolari da deficit di collagene VI, e (iii) la CiP-D e il PTP possono essere considerati validi bersagli farmacologici per la terapia di queste distrofie.

Il secondo obiettivo del lavoro è stato quello di valutare gli effetti terapeutici su topi *Col6a1*^{-/-} di un derivato della CsA, la D-metilalanina³-etilvalina⁴-ciclosporina o Debio 025, il quale mantiene la proprietà di inibire le ciclofiline, ma non la calcineurina. Abbiamo, quindi, studiato, la traslocazione calcineurina-dipendente di NF-AT, l'attivazione dei linfociti T, la probabilità di apertura del PTP in mitocondri isolati e fibre muscolari, ultrastruttura e grado di apoptosi spontanea a livello dei muscoli scheletrici, prima e dopo la somministrazione, a topi *Col6a1*^{-/-}, di Debio 025. I risultati ottenuti hanno dimostrato che il trattamento con Debio 025: (i) non inibisce la calcineurina, ma ha potere desensibilizzante sul PTP; (ii) previene la disfunzione mitocondriale, normalizza i livelli di apoptosi spontanea e i difetti ultrastrutturali dei topi distrofici. Quindi, la desensibilizzazione del PTP può essere ottenuta grazie all'inibizione della CiP-D senza l'azione sulla calcineurina. Questi dati sono la prova che il Debio 025 ha effetti terapeutici sul modello animale per le distrofie da deficit di collagene VI e rappresentano, inoltre, un importante passo avanti verso una possibile terapia a lungo termine per i pazienti affetti da UCMD o BM, dato che il Debio 025 è privo di effetti immunosoppressori.

Una terza questione analizzata è stata, infine, se l'anomala depolarizzazione mitocondriale presente nelle cellule di pazienti affetti da deficit di collagene VI fosse una caratteristica distintiva di queste patologie, argomento dibattuto in una recente pubblicazione. Abbiamo, perciò, studiato la risposta mitocondriale nelle colture cellulari primarie ottenute da biopsie muscolari di pazienti UCMD e BM, in risposta a diversi stimoli e in funzione del numero di passaggi in coltura. I nostri risultati indicano che la disfunzione mitocondriale viene persa dopo 7 passaggi in coltura e che, dopo questo tempo, le cellule risultano meno sensibili a diversi stimoli apoptotici. Questi dati sono compatibili con una selezione delle cellule resistenti all'apoptosi, che probabilmente erano già presenti fin dall'inizio in coltura, ma in numero limitato. Quindi, la disfunzione mitocondriale, e in generale la presenza di un fenotipo patologico, dovrebbero essere analizzati in colture giovani; infine, il numero di passaggi è un fattore critico da considerare quando vengono paragonati studi su diversi pazienti e su diverse patologie.

INTRODUCTION

1. Pathways of cell death

The most widely recognized classification of mammalian cell death includes two types: apoptosis and necrosis. Apoptosis and necrosis have peculiar morphological, biochemical, and molecular features. The main difference can be considered the regulation, or unregulation, of the death process. Cell death is “programmed” if it is genetically controlled. Apoptosis is a programmed cell death, while necrosis is traditionally considered an accidental form of death. However, there is now evidence that necrosis can in certain instances be initiated or modulated by programmed control mechanisms. How cell death occurs is determined by the type and intensity of noxious signals, ATP concentration, cell type, and other factors. It would be an oversimplification to consider apoptosis and necrosis only as independent processes; actually they can be correlated and interconnected. Recently, another process has been linked to cell death: autophagy. Autophagy is an adaptive response to stress induced by, for example, nutrient deprivation and hypoxia, that supplies the cell with metabolites to produce ATP. It is a dynamic process in which portions of cytoplasm are sequestered within double-membraned vesicles (autophagosomes) and delivered to lysosomes for degradation and subsequent recycling. Although autophagy can serve as a protective mechanism against apoptosis and starvation by recycling macromolecules and removing damaged mitochondria and other organelles, excessive autophagy results in cell death with appearance of excessive autophagic vesicles [109;118]. Moreover, several studies have demonstrated that both excessive and defective autophagy can be detrimental for the cell, confirming the existence of an intricate interplay between the autophagic and apoptotic pathways [28;114;134].

1.1. Necrosis

Necrosis is morphologically characterized by a gain in cell volume (oncosis), swelling of organelles, plasma membrane rupture and subsequent loss of intracellular contents (Figure 1). This latter process, in particular, can induce an inflammatory response leading to defense and repair mechanisms. Several mediators, organelles and cellular processes have been implicated in necrotic cell death, but it is still unclear how they interrelate with each other. For a long time, necrosis has been considered merely as an accidental and uncontrolled form of cell death, but evidence is accumulating that the execution of necrotic cell death may be finely regulated by a set of signal transduction pathways and catabolic mechanisms. As already mentioned, different types of cell death could be linked together and a hypothesis suggests that when the intensity of the insult is very high and/or when ATP generation is deficient, cells fail to execute apoptosis and this switches to necrosis [111].

1.2. Apoptosis

Apoptosis, which was originally described by Kerr et al. [96], is a form of regulated cell death critical for sculpting tissue during development, and for maintaining homeostasis in the adult when infected, injured or aged cells have to be eliminated. Cells undergoing apoptosis are characterized by specific morphological and biochemical changes including cellular and nuclear shrinkage, as well as chromatin condensation (Figure 1).

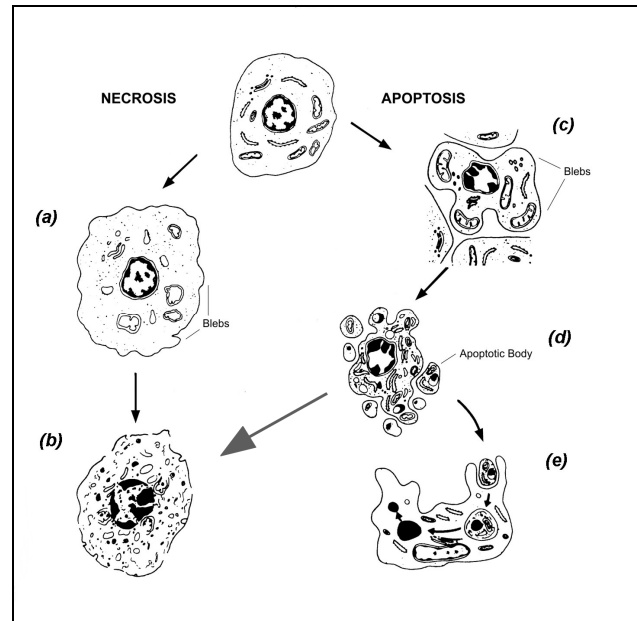


Figure 1. Pathways of cell death: necrosis and apoptosis.

During necrosis the cell forms blebs, swells (a) and releases cytosolic constituents after permeabilisation of the plasma membrane (b), leading to an inflammation reaction in tissues. Apoptosis, also called 'programmed cell death', is characterised by several specific morphological and biochemical aspects that are different from necrosis. An apoptotic cell shrinks, forms blebs, and detaches from its neighbours, while the plasma membrane and cellular organelles remain intact (c). In the nucleus, the chromatin condenses at the nuclear membrane. Finally, the cell disintegrates into apoptotic bodies (d) that are taken up by neighbouring cells (e). The two types of cell death can be linked and apoptosis can switch to necrosis when the stimulus is acute or the ATP is deficient. From <http://www.imm.ki.se/sft/text/eneews4.htm>.

Classically, two major pathways of apoptosis are distinguished, based on the origin of the apoptotic signal. The extrinsic pathway, in which the signal is delivered through cell surface transmembrane receptor-mediated interactions, and the intrinsic pathway, which involves mitochondria and is triggered by a wide variety of extracellular and intracellular stimuli [104].

In the extrinsic pathway, death ligands (such as members of the tumor necrosis factor receptor superfamily) interact with their cognate death receptors, inducing their oligomerisation. The following recruitment of adaptor proteins to the corresponding death domain motifs on the receptor cytoplasmic region causes the formation of the death-inducing signaling complex (DISC) and the subsequent activation of a family of proteases, called caspases. The name caspase is a contraction of cysteine-dependent asparsartate specific protease [2]; indeed, their enzymatic properties are governed by the use of a cysteine side chain for catalyzing peptide bond cleavage and by a stringent specificity for substrates containing aspartate. Caspases recognize substrates containing consensus

sequence (W/Y/I/L/D/V)EXD↓, where X is any amino acid, and ↓ denotes the site of cleavage [178]. Three major groups of caspases have been identified: two are involved in apoptosis, named initiator and effector caspases, and another one is involved in cytokine activation, named pro-inflammatory caspases. Once synthesized each caspase is maintained in a latent state as zymogen (procaspase) such that the apoptotic program is ready to trigger following an appropriate stimulus. The activation of the procaspase can occur by different mechanisms: the initiators are activated by homodimerization facilitated by adapter proteins; The effectors by proteolytic cleavage carried out by the initiators. The procaspase is a single-chain protein, with N-terminal prodomain preceding the conserved catalytic domain; During activation the catalytic domain is cleaved into a large (17–20 kDa) and a small (9–12 kDa) subunit that interact with each other to carry out their function (Figure 2). Another mechanism of activation consists of the formation of a protein complex between caspases and other apoptogenic proteins (e.g. caspase-9 with cytochrome c, see below). Once activated the effector caspases proteolytically cleave a range of substrates leading to the dismantling of the cell.

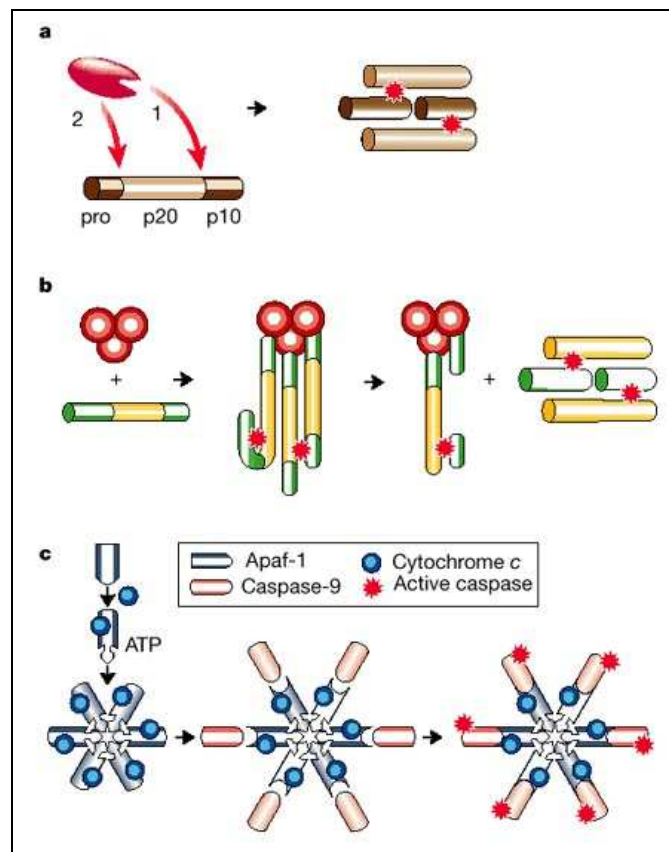


Figure 2. Mechanisms of caspase activation.

Caspases can be activated by different mechanisms. (a) Effector caspases are activated by proteolytic cleavage carried out by other caspases (initiator), (b) initiator caspases are activated by proximity, they form homodimers thanks to other adapter proteins, (c) caspase-9 is activated by the formation of a complex with apoptogenic proteins, cytochrome c and Apaf1 (apoptotic protease-activating factor 1). From [78].

While in some cell types the amount of activated caspase-8 is sufficient to lead to the execution phase of apoptosis (type I cells), this is not the case for others. Indeed, in type II cells (e.g. hepatocytes) the activation of the effector caspases depends on the further

cleavage of the pro-apoptotic Bcl-2 family member Bid to form truncated Bid (tBID) [115], which acts on mitochondria to cause the release of apoptotic factors, thus linking the extrinsic to the intrinsic pathway [52;104;135] (Figure 3).

The intrinsic pathway transduces many different stimuli, including loss of survival/trophic factors, toxins, radiation, hypoxia, oxidative stress, ischaemia-reperfusion, and DNA damage. The downstream signaling for each of these stimuli converges on the central death machinery located at the **mitochondria**. The key event in the mitochondrial pathway of apoptosis is the permeabilization of the outer mitochondrial membrane with the consequent release, in the cytoplasm, of apoptogenic proteins usually locked up in the mitochondria. The proposed mechanisms that cause this event are mainly two: the first depends on members of the Bcl-2 family of proteins, while the second involves the permeability transition pore. Both mechanisms will be described below.

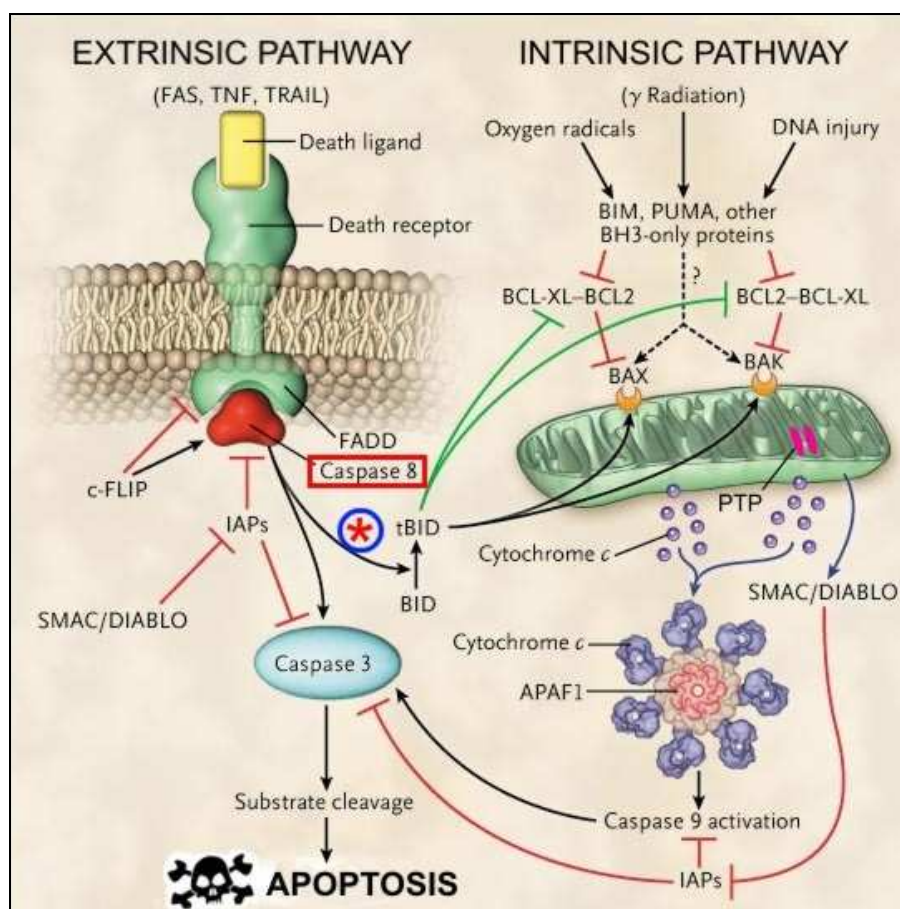


Figure 3. Intrinsic and Extrinsic Apoptotic Pathways.

In the intrinsic pathway pro-apoptotic BH3 proteins of the Bcl-2 family are activated by noxious stimuli, and inhibit anti-apoptotic proteins (BCL2 or BCL-XL). Thus, BAX and BAK are free to induce mitochondrial permeabilization with release of cytochrome c, which ultimately results in the activation of caspase-9 through the apoptosome. The release of cytochrome c can be induced also by the opening of the Permeability Transition Pore (PTP). Caspase-9 then activates caspase-3. SMAC/DIABLO is also released after mitochondrial permeabilization and acts to block the action of inhibitors of apoptosis protein (IAPs), which inhibit caspase activation. In the extrinsic pathway, ligation of death receptors recruits the adaptor protein FAS-associated death domain (FADD) to form the death-inducing signaling complex (DISC). This in turn recruits caspase-8, which ultimately activates caspase-3, the key effector caspase. Cellular FLICE-inhibitory protein (c-FLIP) can either inhibit or potentiate binding of FADD and caspase-8, depending on its concentration. There is a cross-talk between the two pathways (asterisk), which is mediated by the truncated form of BID (tBID) that is produced by caspase-8 mediated BID cleavage; tBid acts to inhibit the BCL2-BCL-XL pathway and to activate BAX and BAK. APAF1 denotes apoptotic protease-activating factor 1, BH3 BCL homologue, TNF tumor necrosis factor, and TRAIL TNF-related apoptosis-inducing ligand. From [80].

2. Mitochondria

Mitochondria are essential hubs for the cell, participating in energy conversion, regulation of signaling cascades and apoptosis. These organelles possess three distinct compartments: the intermembrane space, the intracristal space, and the matrix, defined by two membrane systems. The outer mitochondrial membrane (OMM), which separates mitochondria from the cytoplasm, and the inner mitochondrial membrane (IMM), where the key enzymes of oxidative phosphorylation are localized. The IMM is tightly packed into the *cristae*, which are identified as invaginations of the inner membrane. The *cristae* form “bags” and delimit an intracristal space connected to the intermembrane space by a bottleneck (the *crista* junction). The OMM and the IMM are significantly different from each other in many ways, both structurally, for the composition of the phospholipid bilayers, and for the properties resulting from it, that is permeability. The OMM is much more permeable as a result of the abundant presence of the voltage-dependent anion channels (VDACs), a 3-member protein family which forms large channels (about 2-3 nm in diameter) and allows ions, metabolites, and small molecules to move between the cytoplasm and mitochondria. In contrast, the IMM is highly impermeable, and the transport of ions, molecules, and metabolites is mediated by specialized membrane channels, transporters and exchangers. The IMM is a very good electric insulator: at a thickness of about 10 nm, this membrane can keep a potential difference of >180 mV [132]. Proton pumping driven by substrate oxidation constantly supports the formation of the proton electrochemical gradient ($\Delta\tilde{\mu}_{\text{H}^+}$), which is composed by the sum of the membrane potential ($\Delta\psi_m$) and pH differentials (ΔpH). The proton gradient provides the driving force for ATP synthesis and for ion and metabolite transport.

2.1. Role of mitochondria in energy production

Mitochondria are “energy-converting organelles”, because most of the cell energy conversion indeed takes place in the mitochondria by the oxidative phosphorylation process, which couples substrate oxidation to ATP synthesis. The three major processes eventually leading to ATP synthesis are: (i) the tricarboxylic acid (TCA) cycle, located in the matrix, in which NADH and FADH₂ are produced from organic compounds, (ii) the mitochondrial respiratory chain, in which electrons deriving from NADH and FADH₂ are sequentially transferred to oxygen by electron carriers, the respiratory chain complexes, and (iii) the phosphorylating system (F₁F₀-ATPase), which uses the energy supplied by the respiratory chain in the form of the proton gradient to catalyze the formation of the chemical bond between ADP and P_i. As previously mentioned, the key enzymes of oxidative phosphorylation are located in the IMM. The respiratory chain is made of four distinct IMM-associated multi-polypeptide complexes (complexes I to IV), three of which (complexes I, III and IV) act as oxidation-reduction-driven proton pumps able to create the $\Delta\tilde{\mu}_{\text{H}^+}$ exploited by ATPase to synthesize ATP (Figure 4). Complex I (NADH-ubiquinone oxidoreductase) catalyzes the transfer of two electrons from NADH to Coenzyme Q (CoQ) in a reaction associated with proton pumping across the membrane.

This reaction is inhibited by the drug rotenone. Electrons can be also directly transferred from succinate to CoQ, as part of the TCA cycle, at the level of complex II (succinate dehydrogenase). Electrons are transferred by complex III (ubiquinone-cytochrome c reductase) from reduced CoQ to cytochrome c. This complex is closely associated in varying proportions with complexes I and IV to form a supercomplex called respirasome [1] and can be inhibited by antimycin A. The final step in the electron transport chain is the sequential transfer of 4 electrons from reduced cytochrome c to O_2 . The final reduction of O_2 to H_2O is catalyzed by complex IV (cytochrome c oxidase) and is inhibited by KCN. The F_1F_0 -ATPase is a multisubunit complex located in the IMM whose catalytic part (F_1) and lateral stalk protrude into the matrix compartment, while the F_0 moiety and the remaining portion of the lateral stalk are embedded into the IMM [1;49;189]. The F_1 contains three catalytic sites which cooperate in the synthetic reactions and can be inhibited by aurovertin. The F_0 portion, that can be inhibited by oligomycin, contains the proton channel, and the flow of protons through it generates a torque which is transmitted to F_1 by an asymmetric shaft, the coiled-coil γ -subunit. This acts as a rotating “cam” within the F_1 , sequentially releasing ATP from the three active sites. When $\Delta\tilde{\mu}_{H^+}$ is favorable, the ATPase catalyzes ATP synthesis coupled to spontaneous H^+ flux toward the matrix compartment, but the reverse reaction (hydrolysis of ATP and proton pumping in the intermembrane space) is also possible in certain conditions.

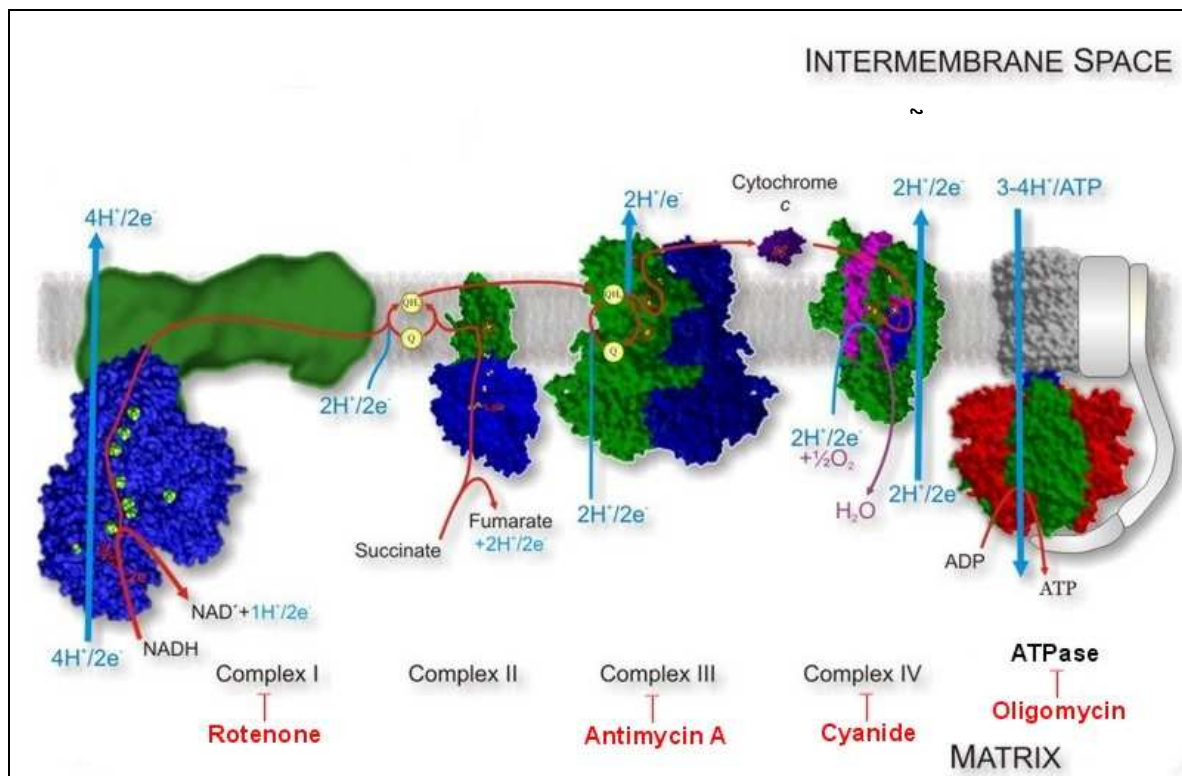


Figure 4. The respiratory chain and the F_1F_0 ATP synthase.

The figure shows the transfer of electrons through the respiratory chain complexes, the proton extrusion in the intermembrane space (complex I, III and IV) and the synthesis of ATP by ATPase. The final step of respiration (complex VI) is the reduction of molecular oxygen to water. The energy released in this reaction is conserved in the form of a transmembrane electrochemical gradient of protons across the membrane, which is mostly used by the ATPase for formation of ATP. In red are shown some inhibitors of the different complexes. From http://www.biocenter.helsinki.fi/bi/biophys/biophys_research.html.

2.2. Role of mitochondria in cell death

As already mentioned, mitochondria play a key role also in the pathways to cell death. These organelles are involved both in the intrinsic pathway of apoptosis, but also in the extrinsic one, as part of an amplification loop of signals. The key event in the intrinsic pathway, namely the permeabilization of the OMM, causes the release, in the cytoplasm, of mitochondrial proteins, such as Apoptosis Inducing Factor, Smac/Diablo, and the most critical apoptogen, cytochrome c. When in the cytosol, cytochrome c binds to the adapter protein Apaf-1, changing its conformational state. In the presence of adequate concentration of ATP, Apaf-1 then oligomerises into an apoptosome which recruits and activates caspase-9 (effector caspase), that triggers the apoptotic cascade. Nevertheless, this mechanism of release may be more complex; early studies have demonstrated that the majority of cytochrome c stores are located in the intracristal space, while only 10-15% of the protein is located in the intermembrane space [17]. Hence, a controlled step of cytochrome c release is the so-called “*cristae* remodeling”, characterized by fusion of individuals *cristae* and widening of *cristae* junctions. This phenomenon causes the redistribution of the cytochrome c trapped in the *cristae* to the intermembrane space, and the consequent release of larger amounts of the protein across the OMM [168]. At the core of this process are machineries that regulate the shape of the organelle. The main player that participates in the regulation of the remodeling seems to be Optic atrophy 1 (Opal), the only dynamin-related protein found so far in the IMM. Indeed, it has been seen that Opal forms oligomers between adjacent membranes of the *cristae*, maintaining the tightness of the *cristae* junctions, the disassembly of these complexes causes the opening of *cristae* junctions and the redistribution of cytochrome c to the intermembrane space [58]. The mechanisms that lead to the permeabilization of the OMM have been the cause for debate with different proposed models. One of these involves members of the Bcl-2 family. This family can be divided into pro-apoptotic and anti-apoptotic proteins. These proteins contain one or more Bcl-2 homology (BH) domains, which share sequence homology and are important for heterodimeric interactions among members of the Bcl-2 family [41;45]. Most anti-apoptotic proteins (as Bcl-2 and Bcl-X_L) contain BH domains 1-4. However, pro-apoptotic proteins can be divided into two groups according to function and the number of BH domains possessed. Bax and Bak are pro-apoptotic, contain BH domains 1-3 and are known as multidomain pro-apoptotic or effector proteins. The remaining pro-apoptotic members contain only the third BH domain and are known as BH3-only proteins (e.g. Bid). BH3-only proteins act as upstream sentinels of cellular damage and derangement. The relatively numerous pro- and anti-apoptotic members of this family engage in complex interactions with each other to ultimately decide whether a cell will commit to death by controlling permeabilization of the OMM. This event can be caused by the activation of Bax and Bak. Bax proteins can be found as monomers in the cytosol or loosely associated with the OMM when not activated. During the activation process Bax translocates to and inserts into the OMM [25;81;190], while Bak is inserted into the OMM even when not activated [186]. Following activation, Bax and Bak undergo conformational changes, oligomerise and permeabilise OMM forming pores in it, leading to the release of

the contents of the mitochondrial intermembrane space, including cytochrome c, into the cytosol (Figure 5). Given the lethal consequences of Bax and Bak activation, this event is regulated by the anti-apoptotic members of Bcl-2 family that bind to Bax and Bak to prevent their activation. However, this mechanism remains poorly defined [34].

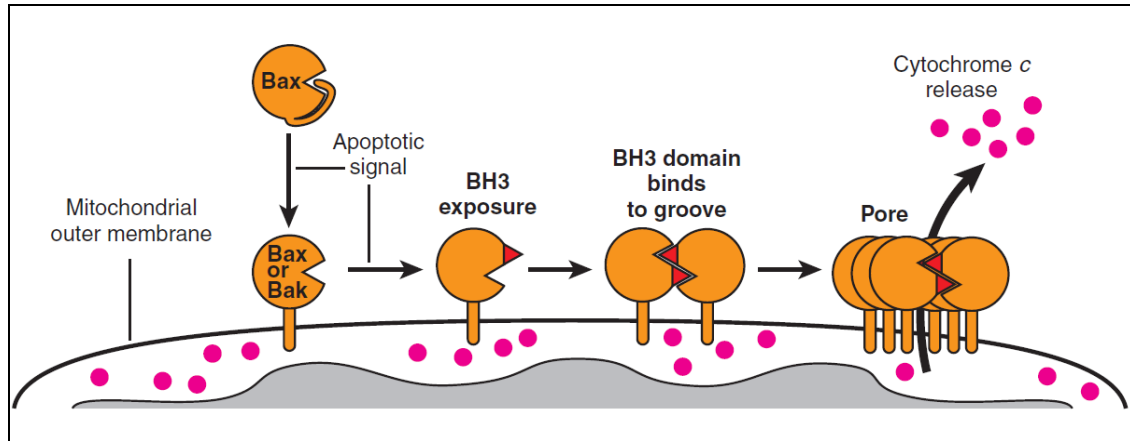


Figure 5. Model of Bax and Bak conformational change and oligomerisation during apoptosis.

Upon apoptotic signalling, the BH3 domain (red triangle) of Bax or Bak is transiently exposed before binding to the hydrophobic surface groove of another activated molecule. Evidence suggests that reciprocal BH3-groove interactions result in the formation of a symmetric homodimer. The dimers presumably then homo-oligomerise via a secondary interface to form large oligomeric complexes that are responsible for pore formation and release of pro-apoptotic factors such as cytochrome c. Note that, in healthy cells, Bak is already anchored in the OMM, while Bax is in the cytosol. Thus, following apoptosis, the initial step in Bax conformational change is the eversion of the domain which anchors it into the OMM. From [46].

Another proposed mechanism suggests that the trigger event leading to the rupture of the OMM may be the opening of the permeability transition pore, which is explained in more detail below. However, it should be kept in mind that these models are not mutually exclusive.

3. Mitochondrial Permeability Transition Pore

The abrupt increase of the IMM permeability, characterized by the sudden loss of $\Delta\Psi_m$, is known as the permeability transition (PT) and the entity responsible for this phenomenon was named the mitochondrial permeability transition pore (PTP). Opening of the PTP (estimated diameter about 3 nm) depolarizes the inner membrane and allows equilibration of ions and solutes up to 1.5 kDa in size between the matrix and the intermembrane space [77]. Respiration is inhibited due to the loss of pyridine nucleotides [48;182]. Moreover, after the PT, the mitochondrial volume may increase when an osmotic imbalance exists, due to the osmotic pressure exerted by non diffusible matrix proteins and macromolecular complexes. Swelling in turn causes matrix expansion and unfolding of the *cristae*, with widening of *cristae* junctions, increased availability of cytochrome c in the intermembrane space and, for long PTP open times, eventual rupture of the OMM and activation of the apoptotic cascade described before (Figure 6). For short open times the PTP does not cause irreversible damage to the mitochondria, while long-lasting openings represent a dramatic event for individual mitochondria. However, in the cell subpopulations of mitochondria exist with different thresholds for opening. Therefore, PTP opening may

occur first in a subset of mitochondria, and either stop there or propagate through the generation of calcium waves and/or of other diffusible signals released by these mitochondria (like reactive oxygen species) which may result in the spreading of the PT to the whole mitochondrial population [71;87;141]. The PTP is a subject of intense study. Despite much research its composition and molecular structure are yet unknown, while its regulatory features have been studied in some detail. The PTP is a high conductance channel of the IMM, with a diameter of 3 nm, when it is completely opened. Its opening is voltage and calcium-dependent, but also many other stimuli and effectors can regulate its state. Long considered as an experimental artifact, the PTP has today acquired a considerable relevance in the pathophysiology of many diseases [19].

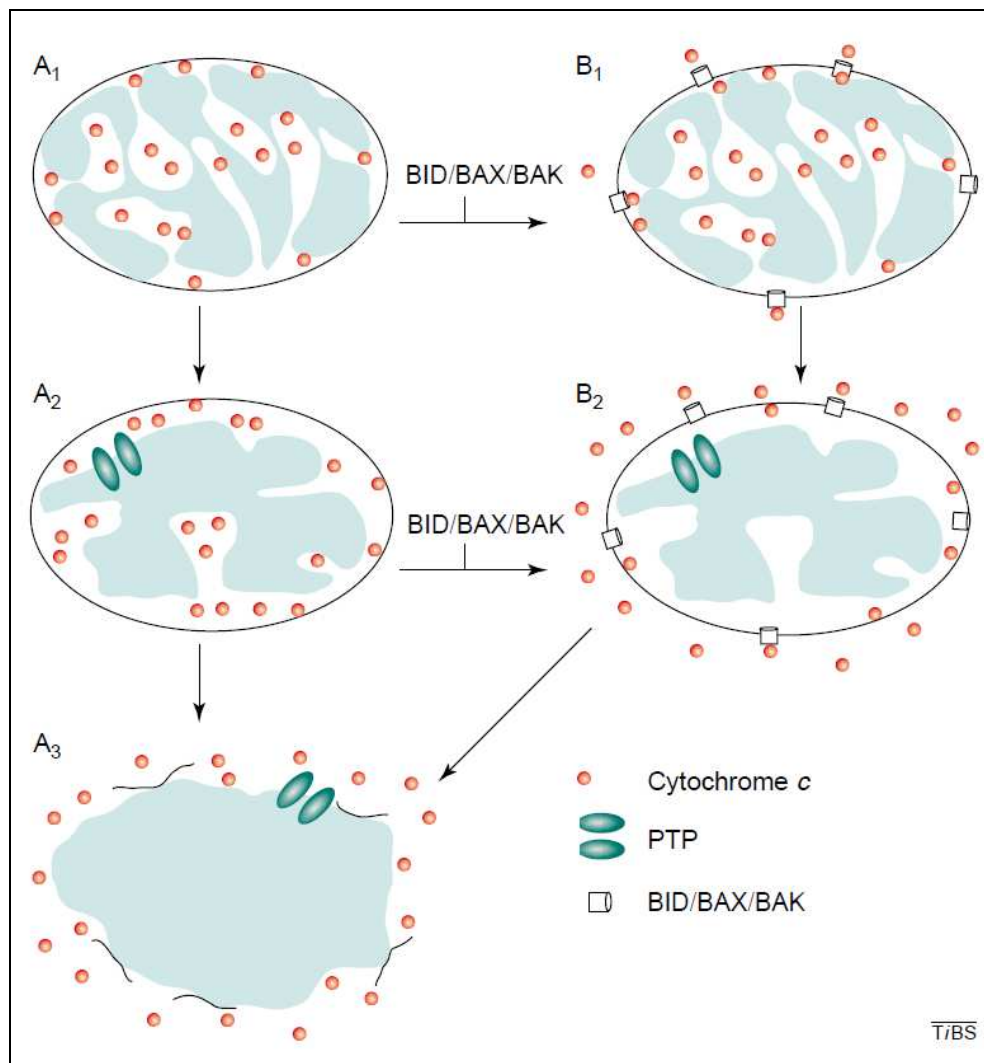


Figure 6. Pathways for mitochondrial swelling and release of intermembrane proteins.

Cytochrome c is compartmentalized within mitochondria in the *cristae* space (A₁), and insertion of BCL2-related proteins (such as BID, BAX or BAK) can result in the release of only small amounts of it (B₁). Depending on whether the BID, BAX or BAK pathway has been activated, PTP opening can cause swelling of the matrix (light blue space), without release of intermembrane proteins (A₂) or with increased release of cytochrome c (B₂). PTP opening could affect the cell fate through ATP depletion and Ca²⁺ dysregulation even in the absence of cytochrome c release (A₃). Stable PTP opening would cause OMM rupture with release of all apoptogenic proteins (A₃). From [20].

3.1. Regulation and structure of the PTP

Many compounds, or conditions, modulate the pore open-closed state; many drugs also affect it, suggesting various hypotheses about its molecular structure (Figure 7).

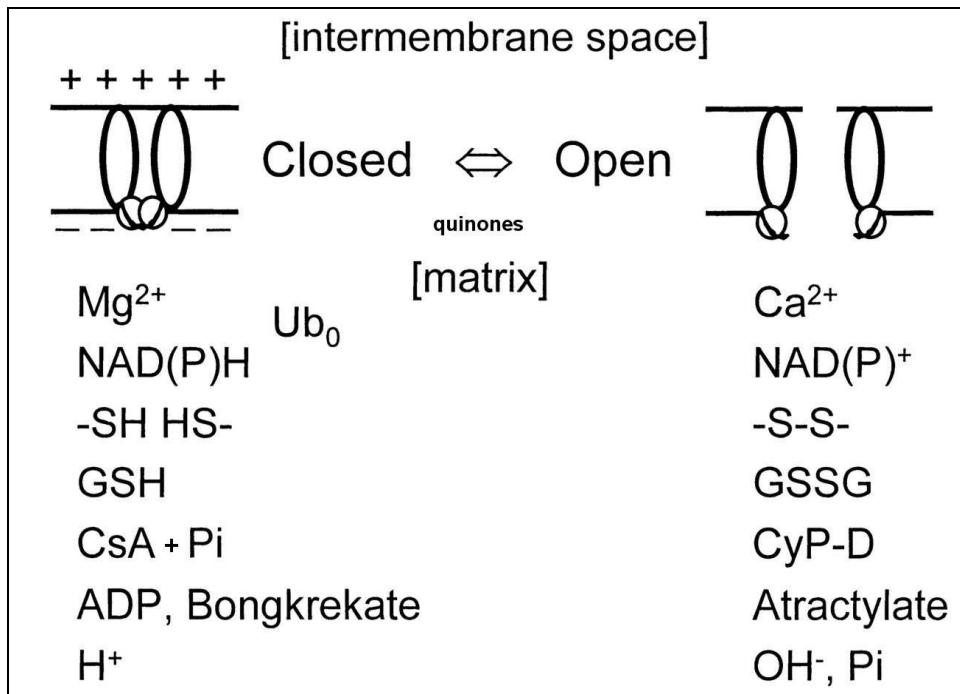


Figure 7. Regulators of the Permeability Transition Pore (PTP).

The probability of PTP to open is influenced by several compounds or conditions. Signs denote transmembrane electrical potential. Ub₀, ubiquinone 0; CsA, cyclosporin A. CyP-D, cyclophilin D. Modified from [16].

There are PTP inducers, that favor the open conformation and increase the probability to open of the PTP, and PTP inhibitors that do not block permanently the opening of the PTP, but only delay it (actually it is more appropriate to define their effect as a “desensitization” than as an inhibition). Here mentioned are some of the classical regulators of the PTP:

- Matrix pH, where the optimum pH for PTP opening is -7.3, while at both lower (through a mechanism involving reversible protonation of histidyl residues), and higher pH values (unknown mechanism), the open probability decreases [140].
- IMM potential, a drop of the membrane potential causing PTP opening, whereas a higher (inside negative) potential stabilizes the pore in its closed conformation [15].
- Ca^{2+} is an essential permissive factor for PTP opening. Ca^{2+} is unique in that all other divalent metals, such as Mg^{2+} , Sr^{2+} , and Mn^{2+} instead decrease the PTP open probability. However, induction of the PT by Ca^{2+} is dependent on additional factors, collectively termed “inducers”.

- Phosphate, the inducing effect is due to the fact that an increase of P_i produces a consequent decrease of matrix $[Mg^{2+}]$, a buffering of matrix pH at ~ 7.3 , and may generate polyphosphate, all conditions that promote PTP opening. Nevertheless, this inducing effect is not specific for P_i , in fact it is shared also by its analogs arsenate and vanadate. It has been recently demonstrated by our group that P_i can act also as a PTP inhibitor when cyclophilin D is ablated or Cyclosporin A is added. The effect is specific for P_i which is able to bind a PTP regulatory site and carries out a desensitizing effect on the pore (Figure. 8) [14].

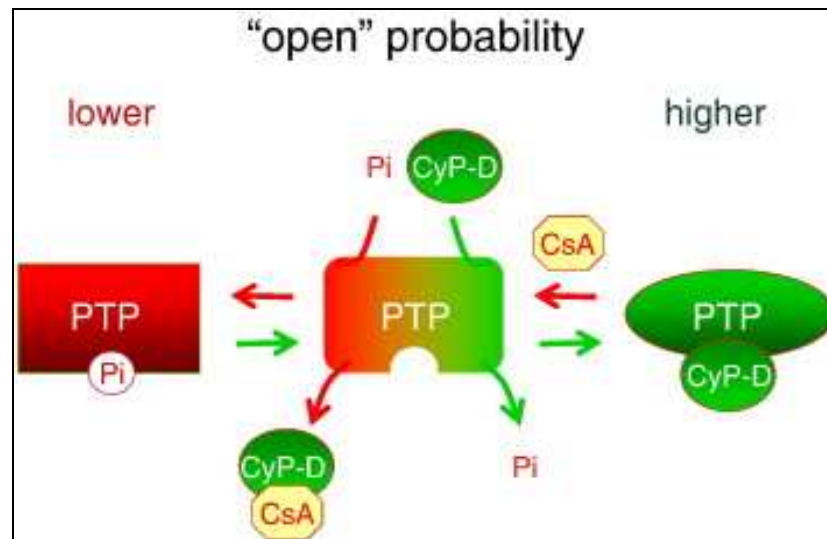


Figure 8 . Modulation of the permeability transition pore by CyP-D and P_i .

The PTP displays a higher probability of opening (right) when it binds to cyclophilin D (CyP-D); addition of Cyclosporin A (CsA) displaces CyP-D and unmasks a cryptic site for P_i (center); if the P_i concentration is sufficiently high, P_i binding decreases the probability of pore opening (left), in a reaction that can be readily reversed if the P_i concentration decreases. From [68].

- Bongkrekate and atractylate; both compounds are inhibitors of the most abundant protein of the IMM, the adenine nucleotide translocator (ANT). They cause an opposite effect on the PTP, bongkrekate being an inhibitor and atractylate an inducer [83;84].
- Ubiquinone 0 (Ub0), is a potent PTP inhibitor, although the binding site on the pore remains undefined. Other quinones may act as inhibitors or inducers through effects whose relation with quinone structure has not been solved despite a large number of studies [184].
- Cyclosporin A (CsA), is a potent immunosuppressant used to prevent transplant rejection, and the most specific desensitizer of the PTP [31;44]. The mechanism through which CsA affects the PTP appears to be indirect, in the sense that the drug does not directly cause PTP desensitization, but rather unmasks a cryptic inhibitory site for P_i (Figure 8).

Many hypotheses have been proposed over the years on the molecular nature of the PTP, yet the composition of this pore remains a mystery. Here I shall review the putative components of the PTP.

3.1.1. ANT

ANT is a relatively small protein (about 300 amino acidic residues), that catalyses the selective and reversible exchange of ADP for ATP in the IMM. The direction of the nucleotide transport is determined by the membrane potential and the adenine nucleotide gradients. Under physiological conditions ATP⁴⁻ is transported out of mitochondria and ADP³⁻ is transported into the mitochondria resulting in membrane depolarization. In the absence of a respiration-driven membrane potential glycolytic ATP is instead taken up, to fuel the ATP synthase running in reverse. ANT is able to transport also other molecules, such as phosphoenolpyruvate, pyrophosphate, and creatine phosphate, although with very low efficiency. Based on the effect of ANT inhibitors (bongkrekate and atractylate) on the PTP, it was originally suggested that ANT might be a core element of the PTP complex. It was seen that bongkrekate causes the transition of ANT into its m-conformation and favors PTP closure, while atractylate promotes the ANT c-conformation, and opens the PTP. However, the transition from m- to c-conformation produces also a change in the membrane potential [158], and this suggests that the effect on the PTP may be indirect [32]. In addition, compelling evidence that ANT is not an essential element of the PTP, was obtained from the analysis of mitochondria isolated from mice lacking the two major isoforms of ANT. The only difference between wild type and ANT^{-/-} mitochondria, was that a larger Ca²⁺ accumulation was necessary to trigger the PT in the null mitochondria, which were expectedly insensitive to ANT ligands, yet could undergo a Ca²⁺-dependent and CsA-sensitive PT with matrix swelling [101]; thus, a PT can occur in the absence of the ANT.

3.1.2. VDAC

VDAC is the most abundant protein of the OMM, and in mammals there are three isoforms of this protein. The VDAC conductance is the major factor in regulation of the mitochondrial inward and outward traffic of substrates, nucleotides, amino acids, small peptides, signaling molecules, etc. [112]. The hypothesis that VDAC could be part of the PTP derives from the finding that VDAC channels have a diameter of 2.5- 3 nM, and electrophysiological properties similar to those of the PTP [173;175]. Moreover, some VDAC regulators have also an effect on the PTP, such as NADH, Ca²⁺, and hexokinase [66;147;197]. However, the main features of the PT are still preserved in mitochondria isolated from VDAC1^{-/-} [102] and VDAC3^{-/-} mice, and in VDAC1-3^{-/-} cells in which VDAC2 has been silenced [10]. Thus, VDAC is not a PTP core element and currently there is no compelling direct evidence for its PTP-regulatory role either.

3.1.3. Hexokinase

Hexokinase (HK) is the first enzyme of glycolysis. It provides a link between mitochondria and cytosol by governing the preferential utilization of mitochondrial ATP

for the conversion of glucose to glucose-6-phosphate. In mammals, there are four isoforms of HK (I to IV). HK II appears to be the principal regulated isoform in many cell types and its expression is significantly increased in aggressive tumors, where it is involved in the increased glycolysis allowing cells to grow in the hypoxic conditions of the growing tumor. HK I and HK II are able to bind to VDAC through their N-terminal sequences [8], and the enzyme association/dissociation regulates the movement of HK from mitochondria to cytosol [194]. Furthermore, HK II maintains the integrity of the OMM and the binding between VDAC and HK is thought to prevent apoptosis via several mechanisms. Recent findings indicate that forced release of HK II from mitochondria, by drugs (eg. clotrimazol) or peptides (corresponding to the N-terminal domain), generates a cell death signal that engages PTP opening. Surprisingly, this peptide was also fully effective in *VDAC1,3^{-/-}* fibroblasts (*VDAC2* does not bind HK) proving the dispensability of VDAC for the induction of apoptosis in these cells [40].

3.1.4. Peripheral benzodiazepine receptor

The peripheral benzodiazepine receptor (PBR) is a OMM hydrophobic protein, initially identified as a binding site for benzodiazepines in tissues that lack GABA receptors (i.e. the clinical target of benzodiazepines in the CNS). The PBR is present in a wide variety of tissues, and particularly abundant in cells producing hormones, where it promotes the transport of cholesterol into the mitochondrial matrix for steroid synthesis [144]. Other functions assigned to this receptor include porphyrin transport [181] and oxygen (redox) sensing [193]. The first suggestion that PBR could be involved in PTP function derived from the finding that PBR was able to form a multimeric complex with VDAC and ANT [122]. Additionally, patch clamp studies have revealed that the PTP was sensitive to the benzodiazepine Ro5-4864 (i.e. a PBR ligand) [99] and that this drug, administered at the onset of reperfusion, protected against post-ischemic damage, probably acting on a target which is located upstream of PTP, thus suggesting that PBR is probably a regulator, but likely not to be a PTP component [33]. The examination of mitochondria lacking the PBR would greatly help clarify these issues, but initial attempts to generate such animals result in embryonic lethality [144].

3.1.5. Phosphate carrier

The phosphate carrier (P_iC) is a member of the mitochondrial carrier family and catalyzes the transport of P_i across the IMM. Uptake of P_i into mitochondria is essential for phosphorylation of ADP to ATP. A role for this mitochondrial transporter in the PTP composition was recently hypothesized based upon evidence that compounds, such as N-ethylmaleimide (NEM), Ub0 and Ro 68-3400, which all inhibit PTP opening [38;56;150] are also P_iC inhibitors [113]. However, this evidence was obtained in de-energized mitochondria incubated in the presence of 40 mM P_i , which casts doubts on its relevance under physiological conditions. This argument holds especially true for the relationship between P_i and Ca^{2+} uptake in mitochondria. In respiring mitochondria P_iC inhibition inevitably affects mitochondrial Ca^{2+} uptake. By blocking P_i uptake NEM limits Ca^{2+}

uptake to levels that are below the threshold for PTP opening [138], so that it would be difficult to attribute PTP inhibition to decreased P_i rather than Ca^{2+} uptake. At variance from NEM, neither Ub0 [56] nor Ro 68-3400 [38] inhibit P_i transport at concentrations that fully inhibit the PTP as shown by the total lack of effect on mitochondrial Ca^{2+} uptake; and, perhaps more importantly, these ubiquinone analogues desensitize the PTP to Ca^{2+} even when P_i is replaced by arsenate or vanadate [14]. Whether the P_iC takes part in PTP formation should be addressed by the same genetic approaches exploited for elucidating the role of ANT and VDAC [10;101;102].

3.1.6. Cyclophilin D

Cyclophilin (CyP)-D is the mitochondrial isoform of Cyclophilins, a large family of chaperones, that are endowed with peptidyl-prolyl *cis-trans* isomerase (PPIase) activity [53;176]. Its involvement in the formation of the PTP was suggested based on the observation that CsA (inhibitor of all CyPs) has a desensitizing effect on the PTP [31;44;57;70;174]. Several genetic studies have demonstrated that CyP-D is a regulator rather than a component of the pore [9;13;136;165].

4. Cyclophilins

These isomerases are ubiquitously distributed within the compartments of the cell and highly conserved throughout evolution suggesting the importance of their enzymatic activity. In the proteins that contain proline, the CyPs stabilize the *cis-trans* transition state and accelerate the isomerization of the bonds preceding proline (peptidyl-prolyl bonds), a process that is considered important not only in protein folding but also during the assembly of multidomains proteins (Figure 9).

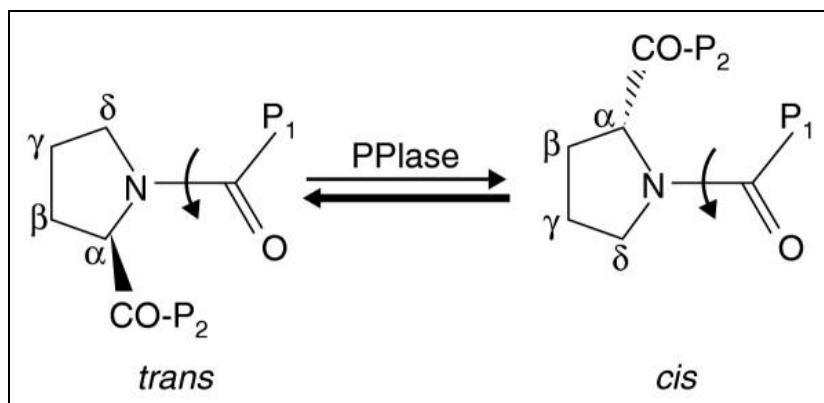


Figure 9. Schematic illustration of the reaction catalyzed by the cyclophilins.

In the figure are shown the *trans* and *cis* isomers of the peptide bond between proline (on the left of each structure shown) and another amino acid (P_1 , on the right). The interconversion between the two forms is catalyzed by cyclophilins and other peptidyl-prolyl isomerases (PPIases). The carbon atoms of the proline are indicated by Greek letters; P_2 indicates a third amino acid on the other side of the proline. The peptide bond has some double-bond character and is planar. From [185].

Sixteen different CyPs have been found in humans; all share a common domain of about 109 amino acids, the CyP-like domain, surrounded by domains unique to each member that are associated with subcellular compartmentalization and functional specialization.

The archetypal CyP, CyP-A, is localized in the cytosol of all tissues, while CyP-B and CyP-C are in the endoplasmic reticulum and CyP-E in the nucleus. As already mentioned, CyP-D has a signal sequence that directs it to mitochondria [3;73].

4.1. CyPs in diseases

In recent years, CyPs have been demonstrated to play a role in a variety of pathophysiological processes. The existence of tissue- and organelle-specific isoforms, moreover, explains the diversity of pathways that CyPs are involved in. CyP-A, for instance, has been demonstrated to contribute to the inflammatory processes in rheumatoid arthritis [97], to mediate endothelial activation [98], and to be involved in the innate immunity to HIV [171]. Many studies have shown the role of CyP-B in hepatitis C infection [54]. Furthermore, the expression and the functions of CyPs have been found to correlate with several types of cancers including pancreatic carcinoma [192]. Thus, understanding the role of CyPs in these conditions could be crucial in determining clinical applications for the treatment or diagnosis of human diseases.

4.2. Cyclosporin A and its analogues

All CyPs are able to bind the immunosuppressive drug CsA, a cyclic undecapeptide produced by the fungus *Tolypocladium inflatum*; CyP-A, in particular, is the major intracellular receptor for CsA [74]. As already mentioned, CsA is able to affect PTP opening through an unknown mechanism that involved CyP-D. However, CsA has many different effects since acts on all the CyPs. For instance, the mechanism through which CsA carries out its immunosuppressive effect involves CyP-A. Following formation of the complex between CsA and CyP-A, this binds to and inhibits calcineurin, a calcium-calmodulin-dependent phosphatase (Figure 10). This protein couples intracellular calcium to dephosphorylation of specific substrates resulting in diverse biological consequences depending on cell type; for example, calcineurin is involved in neuronal growth, development of cardiac valves and hypertrophy, and activation of lymphocytes. Thus, the inhibition of calcineurin by CsA causes several effects, first and foremost the block of the immune response against transplants. Indeed, when calcineurin is inhibited, it no longer dephosphorylates nuclear factor of activated T cells (NF-AT), which remains in its phosphorylated (inactive) form in the cytosol. The failed translocation of NF-AT to the nucleus prevents the activation of the transcription of genes encoding cytokines, such as interleukin-2, and thus of the immune response against transplants. It should be noted that calcineurin plays a key role also in skeletal muscle differentiation, regeneration, and fiber type conversion to an oxidative state, all functions that are crucial to muscle development, metabolism, and functional adaptations [12]. Another substrate of calcineurin is the pro-fission GTPase dynamin-related protein (Drp) 1 [43]. Drp1 is believed to provide the mechanical force to fragment mitochondria during apoptosis, but to carry out its function it has to move from the cytosol to the organelles. Like NF-AT, Drp1 has to be dephosphorylated by calcineurin to translocate to mitochondria. Inhibition of calcineurin by CsA blocks the dephosphorylation of Drp1 and mitochondrial

fragmentation [37]. This discovery underlines an important issue, namely that CsA affects mitochondrial shape and function also independently of its interaction with CyP-D [37], a fact that should be considered during the evaluation of the mitochondrial effects of this drug.

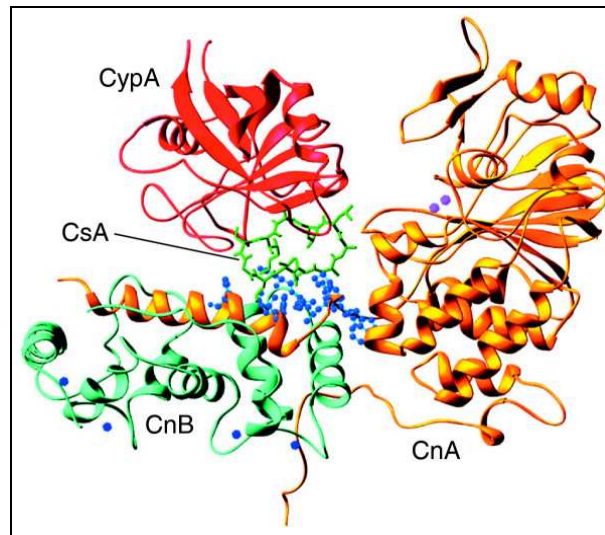


Figure 10. Crystal structure of the complex between Cyclosporin A(CsA)/Cyclophilin A (CyP-A)/calcineurin. The CsA/CyP-A binary complex lies at the base of the helical arm of the catalytic subunit of calcineurin (CnA) that binds the regulatory subunit calcineurin (CnB); it nestles in a hydrophobic groove in intimate contact with both subunits, at a region unique to calcineurin and not found in other phosphatases, and this intimate contact gives the interaction high specificity. From [82].

As already mentioned, the CyPs have a key role in many diseases, so CyP inhibitors have been studied for a possible therapeutic utilization. There is a widespread agreement that also mitochondria, and PT, are involved in the pathogenesis of many diseases (see paragraph 5), and thus CsA could be used in these settings. Many chemical Cs-analogues have been developed to improve their potency, and to avoid the immunosuppressive effects, the weightiest side effect of CsA treatment and the reason why this drug could be not employed in chronic or long-term therapies other than transplant rejection itself. The binding domains for CyPs and calcineurin are located at different sites on the CsA molecule, and small structural alterations can reduce the binding to calcineurin while preserving high affinity to CyPs [183]. NIM 811 and DEBIO 025 (originally named UNIL 025, [75]) are two examples of Cs-analogues used in several published studies [121;131;155;155;156;177]. NIM 811 and DEBIO 025 have the same structure of CsA with a modification at the fourth amino acid (Figure 11). Changes at residue 4 are within the critical binding site to calcineurin, and are able to abolish the immunosuppressive activity without reducing the CyP affinity. DEBIO 025 has another modification at residue 3 that increases its affinity for CyP binding.

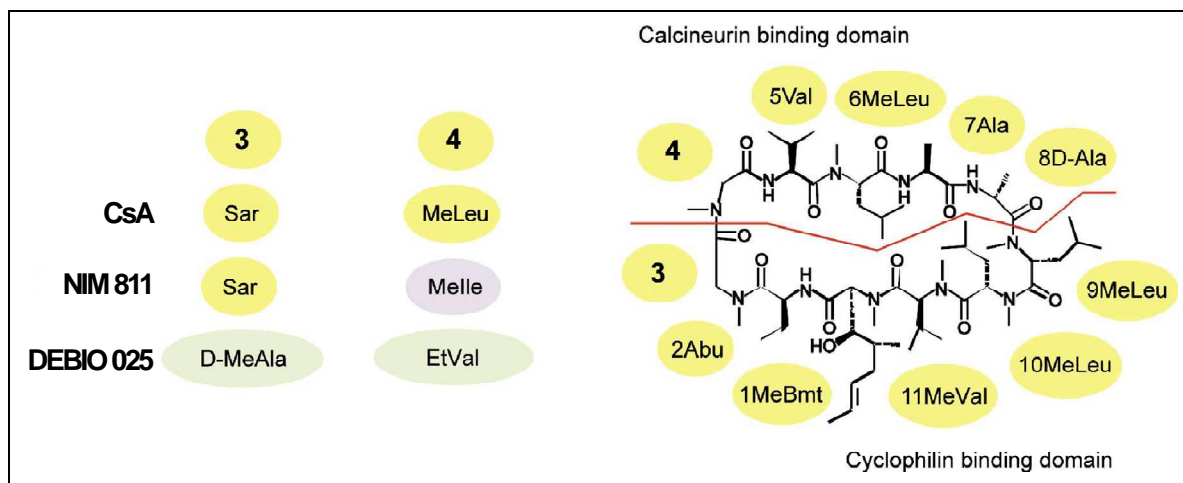


Figure 11. Amino acid sequences for the undecapeptides cyclosporin A (CsA), NIM 811 and DEBIO 025.

The binding site for the cyclophilins to the immunosuppressive CsA molecule covers residues 1–3, 10, and 11, and the interaction with calcineurin residues 4–7. The non immunosuppressive cyclosporin molecules NIM 811 and DEBIO 025 are both modified (as compared to CsA) at the fourth amino acid (from N-methyl-leucine to N-methyl-isoleucine [Melle] and N-ethyl-valine [EtVal] respectively) preventing calcineurin binding while retaining the ability to interact with cyclophilins. DEBIO 025 has an additional modification at the third amino acid (from sarcosine to N-methyl-D-alanine [D-MeAla]). From [75].

Lastly, Cs-analogues could be used to separately evaluate the effects on CyPs and on calcineurin. It should be noted that CsA is able to bind all CyPs, and therefore that interpretation of results obtained with this drug is not trivial. Recently a CsA derivative targeted to mitochondria has been developed [119], that seems to be selective for CyP-D. The aim was to use this drug in models of disease in which CyP-D and PTP have a key role, in order to discriminate the effects on the mitochondrial isoform over all the others present. Different groups, including our own, have also created animals lacking CyP-D, to evaluate the role of this protein in the PTP [9;13;136;166].

4.3. Mice lacking CyP-D

In order to shed light on the role of CyP-D in the PTP regulation, mice have been created in which the gene encoding for CyP-D (*Ppif*) has been eliminated, resulting in mitochondria devoid of this protein. *Ppif*^{-/-} animals were born at the expected Mendelian ratio, and were otherwise indistinguishable from *wild-type* (*wt*) individuals, suggesting that CyP-D is dispensable for embryonic development and viability of adult life. Accurate behavioral studies of *Ppif*^{-/-} mice have demonstrated an increased anxiety, facilitation of avoidance behavior, and the development of obesity in adults independently of food and water intake [117], suggesting that these animals may develop specific phenotypes with aging. In several studies published in 2005 from different groups [9;13;136;166] liver mitochondria, embryonic fibroblasts, thymocytes and hepatocytes isolated from *Ppif*^{-/-} mice were analyzed, evaluating in particular the response to different apoptotic stimuli. Basso *et al.* showed that the absence of CyP-D does not modify the capacity of the PTP to form and open, consistent with its role as a regulator but not as a core structural component. Indeed, the PTP in CyP-D null mitochondria showed a striking desensitization to Ca²⁺ in that the opening required about twice the Ca²⁺ load necessary to open the pore in *wt*

mitochondria. On the other hand, other stimuli such as Ub0, pH, adenine nucleotides had the same effect on the PTP of *wt* as well of *Ppif*^{-/-}. Moreover, CsA, as expected, had no effect on the *Ppif*^{-/-} mitochondria. These general conclusions are corroborated by studies from the other groups that performed studies also in cells lacking CyP-D. These cells were more resistant than *wt* to oxidative stress (H₂O₂) and increased cytoplasmic Ca²⁺ (induced by A23187, or thapsigargin) [166]. In the studies of Baines *et al.*, the resistance of CyP-D null cells to H₂O₂ was reversed on re-expression of CyP-D but not by re-expression of mutant forms of CyP-D lacking PPIase activity, suggesting that this function of CyP-D is essential for its ability to regulate PTP, but this issue is still debated. However, no difference was observed between *wt* and *Ppif*^{-/-} cells and tissues following the treatment with agents (such as staurosporine and TNF- α) that activate apoptotic pathways dependent on pro-apoptotic members of the Bcl-2 family. In addition, Baines *et al.* and Nakagawa *et al.* both demonstrated that hearts from *Ppif*^{-/-} mice are resistant to the ischemia/reperfusion injury, and Schinzel *et al.* showed that CyP-D-deficient mice displayed a dramatic reduction in brain infarct size after acute middle cerebral artery occlusion and reperfusion. Recently, studies have revealed that CyP-D null mitochondria are able to accumulate the same amount of Ca²⁺ as the *wt* in experiments where P_i had been replaced by arsenate or vanadate; and the same results could be obtained in *wt* mitochondria treated with CsA. These experiments suggest that when CyP-D is absent (because of genetic ablation or of the binding with CsA) P_i can act on its inhibitory site on the PTP thereby delaying pore opening [14]. Finally, our group has discovered that CyP-D interacts with the lateral stalk of the F₁F₀-ATP synthase and regulates its function, with increased ATPase activity in the absence of CyP-D or after CsA treatment. Our hypothesis is that CyP-D release from the ATP synthase activates the enzyme [67].

5. PTP in pathophysiology

Several studies have credited mitochondria, and in particular the PTP, with playing a key role in many different pathologies. Largely through the use of CsA and the study of *Ppif*^{-/-} mice, key advances have been made in understanding the role of PTP, and CyP-D, in the pathogenesis of many diseases.

5.1. Heart diseases

The relevance of mitochondrial dysfunction to the onset of irreversible injury of the heart has prompted numerous studies aimed at defining the involvement of the PTP, especially in the setting of myocardial ischaemia-reperfusion [47]. During ischaemia and reperfusion the metabolism and ionic homeostasis of the heart are profoundly perturbed in a way that greatly favors PTP opening. Firstly, during ischaemia, mitochondria in the myocytes are deprived of oxygen and so they are not able to produce ATP by oxidative phosphorylation; and this leads to a rapid fall in tissue [ATP]. These changes stimulate glycolysis but this is unable to provide sufficient ATP to fuel the beating heart and contraction rapidly ceases. Glycolysis continues to provide some ATP, but as lactic acid accumulates, the intracellular

pH drops progressively activating a cascade of events that ends with an increase of cytosol $[Ca^{2+}]$. Nevertheless, the most critical factor is the degree of oxidative stress rather than calcium overload reached during ischaemia, and that primes the PTP for opening at reperfusion. In fact, ischaemia *per se* does not appear to cause PTP opening, but it creates the conditions for pore opening at reperfusion [72]. Targeting the PTP with CsA has proven to protect from heart injury in different models of ischaemia and reperfusion [6;76], and in acute myocardial infarction [152].

5.2. Liver diseases

Many studies have suggested that PTP could be involved in several conditions of liver damage. For instance, mitochondria isolated from rats fed an ethanol-containing diet were demonstrated to be much more sensitive to PTP opening than their isocalorically fed counterparts [146]; treatment of rats with CsA conferred resistance to the short-term effects of D-Galactosamine plus lipopolysaccharide of *Streptococcus* at doses that would otherwise cause extensive liver damage [172]. Moreover, PTP has demonstrated to be involved in a model of hepatocarcinogenesis. There, the arylamine 2-acetylaminofluorene (AAF) was used because of its capacity to cause the onset of liver tumors preceded by a sequence of alterations that resembles the clinical course of chronic hepatitis evolving in cirrhosis. Our group demonstrated that PTP desensitization during AAF feeding induces a tumor-promoting adaptive response that selects apoptosis-resistant hepatocytes [100]. Another study has shown that PT is involved also in the cholestatic liver injury induced by bile duct ligation; and that a Cs-analogue is able to decrease cholestatic necrosis and apoptosis, suggesting that the PT plays an important role in cholestatic cell death *in vivo* [154].

5.3. Neurological diseases

Many neurological disorders are caused or worsened by mitochondrial dysfunction. Many studies have suggested a possible role for PTP in brain damage based on the protective effect of CsA. CsA protects from brain injury provoked by hyperglycemia [55;116], hypoglycemia [51;59], ischaemia [137;179], trauma [163]. Moreover, *Ppif*^{-/-} mice have a striking decrease of the damage induced by middle cerebral artery occlusion [166].

5.4. Tumors

An involvement of mitochondria and the PTP in cancer has been documented. Tumor cells appear to be more resistant to the rupture of the OMM [69]; VDAC, HKII, PBR and CyP-D were found to be dysregulated in a number of neoplastic tissues and tumor cell lines [167;169]. Recently, our group has demonstrated that the increased resistance to death stimuli seen in different cancer cell models depends on the activation of an ERK-dependent signaling cascade that leads to PTP desensitization [153]. Moreover, drugs used for cancer therapy have mechanisms of action which often affect the PTP indirectly, i.e. antioxidant agents, chemotherapeutics which reduce interaction of antiapoptotic Bcl-2

proteins with mitochondria [29]. Besides, the resistance to chemotherapeutics by some tumors could be related to a reduced Ca^{2+} release from intracellular stores upon apoptotic stimuli [39]. Lastly, PTP has been suggested as a potential target in cancer therapy, based on the discovery that lipids (jasmonates) derived from plants, acting on the PTP, induce selective death of transformed tumor cells [157].

5.5. Muscle diseases

The hypothesis that muscle cell death seen in several diseases paradigms was caused by a mitochondrial Ca^{2+} overload and energy depletion was made about 35 years ago by Wrogemann and Pena [191]. Many dystrophies show common pathological signs, such as a high degree of spontaneous apoptosis in muscles or the presence of mitochondrial alterations in muscle fibers. Unexpected and exciting results were obtained by our group in this field studying Collagen VI-related disorders, a subset of human muscular dystrophies [4;5;88;127]. The hypothesis we made was that the PTP could play a role in the pathogenesis of these muscular diseases. Consistent with this, other groups have recently demonstrated that PTP could be involved also in other myopathies, i.e. Duchenne Muscular Dystrophy and limb-girdle muscular dystrophy [131;156].

6. Extracellular matrix and collagen VI

6.1. ECM

The ECM is an intricate network of macromolecules with several functions. Initially considered an inert scaffolding meant to stabilize the physical structure of tissues and organs, it has been today recognized as an active and complex player in cell physiology [86]. Indeed, it regulates the behavior of the cells that contact it, influencing their development, migration, proliferation, shape, and function. Several adhesion receptors link ECM components with the cytoskeleton of the cells, through anchoring and adhesion complexes. ECM proteins are large and complex, with multiple distinct domains, and are highly conserved during evolution [22;50;85;110]. The two main classes of molecules that make up the ECM are (i) proteoglycans, polysaccharide chains covalently linked to proteins, and (ii) fibrous proteins of two functional types: structural (i.e. collagens), that provide resistance and mechanical force to the cells, and adhesive (i.e. fibronectin and laminin), that connect plasma membrane receptors to ECM components [89]. In tissues undergoing extensive mechanical stress, such as skeletal muscle, ECM has a critical role. Muscle cells are individually surrounded by a basal lamina which interacts with ECM constituents that contribute to their stability during contraction. It is no coincidence that genetic defects of components of the anchoring complexes are amongst the most frequent causes of human muscular dystrophies [123;180]. An important family of ECM proteins is the collagens, which play a dominant role in maintaining the structure of various tissues and also have many other important functions. Collagens are involved in cell adhesion, chemotaxis and migration, and the dynamic interplay between cells and collagens regulates tissue remodeling during growth, differentiation, morphogenesis and wound

healing, and in many pathologic states. Collagens are the most abundant proteins in the human body, and the important roles of these proteins have been clearly demonstrated by the wide spectrum of diseases caused by mutations in collagen genes [89]. More than 25 different types of collagen have been recognized so far. Collagen type VI, for instance, plays a critical role in maintaining normal muscle function and cell integrity [164] and its deficiency is one of the most common causes of congenital muscular dystrophies.

6.2. Collagen VI

Collagen (Col) type VI is a glycoprotein widely distributed in the ECM of skeletal muscle and of several other organs such as skin, cornea, lung, blood vessels, intervertebral disks, and joints [95;120]. ColVI forms a microfibrillar network which anchors the basement membrane to the underlying connective tissue [95] through interaction with several ECM constituents including fibrillar ColI and II, ColIV, fibronectin, biglycan and decorin [27;160;188]. ColVI is formed from monomers, each of three peptide chains: $\alpha 1(\text{VI})$ and $\alpha 2(\text{VI})$, both of 140 kDa in size, and $\alpha 3(\text{VI})$ of 250–300 kDa, all of them encoded by distinct genes (COL6A1, COL6A2 and COL6A3). However, the identification, and an initial biochemical characterization of three new chains (ColVI $\alpha 4$, $\alpha 5$ and $\alpha 6$) has recently been reported [62]. The corresponding genes probably derive from duplication of the common ancestor of the ColVI $\alpha 3$ and of the new α chain genes, followed by additional duplications [61]. While a single transcript is produced by COL6A1, $\alpha 2(\text{VI})$ and $\alpha 3(\text{VI})$ chain have several alternatively spliced variants [7;107]. ColVI is a multimodular protein made of several domains. Each chain contains a short triple helical domain of 335-336 amino acids and two large N- and C- terminal globular ends composed of repeated domains of 200 amino acids each, sharing similarity with the von Willebrand factor type A module [42] (Figure 12A). The building of an extended network is possible through a peculiar and complex pathway of ColVI assembly. The assembly starts inside the cell and consists of the equimolar association of $\alpha 1$, $\alpha 2$ and $\alpha 3$ chains to form a triple helical monomer. The monomers (3 chains) then assemble into dimers (6 chains) and tetramers (12 chains), stabilized by disulfide bonds. The tetramers are secreted and deposited in the ECM where they interact in an end-to-end fashion, among their non-collagenous domains, to form beaded microfilaments [60] (Figure 12B). However, based on the identical size of the collagenous domains of the new α chains compared with that of the $\alpha 3$ chain, it has been hypothesized that these could substitute for the $\alpha 3$ in the formation of the heterotrimers [64].

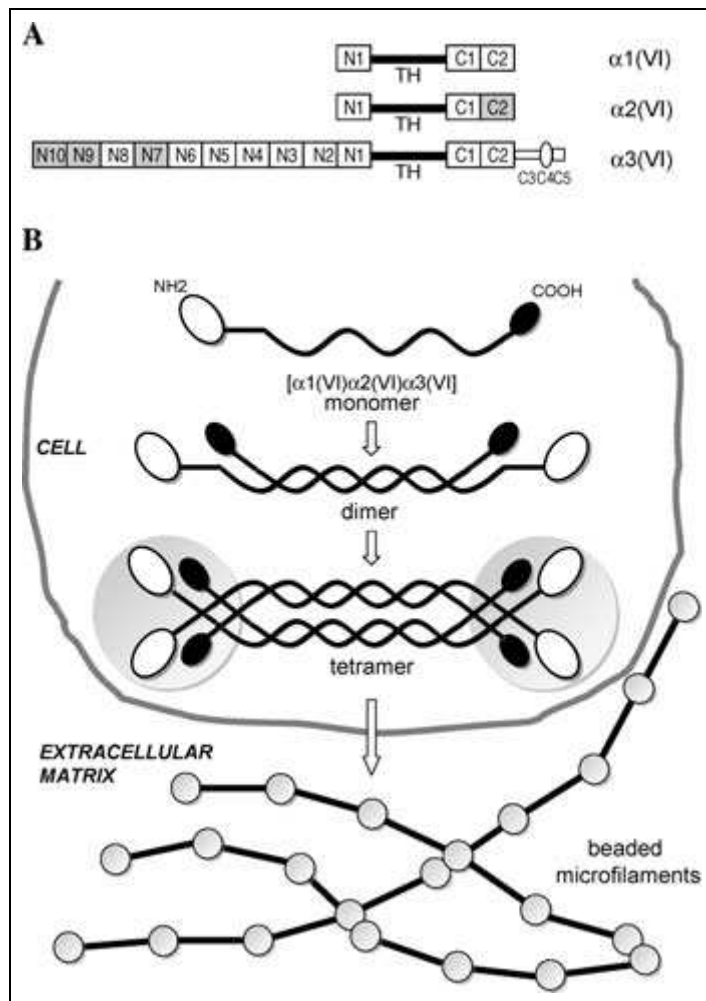


Figure 12. Schematic diagram of ColVI structure and assembly.

(A) Protein domains of $\alpha 1(VI)$, $\alpha 2(VI)$, and $\alpha 3(VI)$ chains. ColVI subunits contain a short triple helical (TH) region and two large N- and C-terminal globular ends composed of domains (N1, C1, C2) sharing similarity with the von Willebrand factor type-A (vWF-A) module. The $\alpha 3(VI)$ chain contains nine additional vWF-A modules (N10-N2) at the N-terminal end, and three peculiar domains (C3-C5) at the C-terminal end. The shaded vWF-A modules have been shown to undergo alternative splicing. (B) Main steps of ColVI intracellular assembly and secretion into the ECM. From [18].

The domains of the ColVI microfibrils form heterotypic interactions with cell surface receptors and other ECM molecules. For instance, the triple helical region promotes adhesion through interaction with several integrins and with the NG2 proteoglycan [35;151]. The ubiquitous expression of ColVI and its interaction with a variety of ECM constituents suggest that it is implicated in the development of the ECM, playing a key role in repair processes and tissue development and homeostasis. ColVI is abundantly synthesized and secreted by skin fibroblasts and smooth muscle cells. In skeletal muscles, ColVI is localized at the level of the endomysium, just outside the basement membrane [103]. Recently, a study has shown that muscle interstitial fibroblasts contribute significantly to the deposition of ColVI in the ECM of skeletal muscle [198], while whether myogenic cells are able to produce it is still debated.

7. Collagen VI-related disorders

Muscular dystrophy is a general term that describes a group of inherited and gradually debilitating myogenic disorders. They are genetically and clinically heterogeneous, but the increased understanding of their molecular basis has allowed for a classification based on the genetic and biochemical defects. ColVI-related disorders are a group of myopathies caused by a deficiency of ColVI, due to mutations of genes that encode the chains of this protein. The main syndromes are Bethlem Myopathy (BM) and the most severe form, Ullrich Congenital Muscular Dystrophy (UCMD). Also other pathological phenotypes have been described which include limb-girdle and myosclerosis variants [128;162]. The severity of the clinical phenotype is, in part, correlated to the amount of ColVI in the ECM, which is generally decreased or apparently normal in BM patients, and much reduced or totally absent in UCMD. However, the phenotype also depends on the quality of ColVI, as in the case of aberrant filaments or proteins with altered functionality [108]. About 70 different mutations of the COL6 genes have so far been associated either with UCMD or with BM. The reason why certain COL6 mutations cause BM and other mutations cause the more severe form UCMD remains obscure. It might be postulated that different COL6 gene mutations may have different impacts on the mRNA and protein level, by predominantly impairing ColVI synthesis, assembly, secretion or function. Based on the type of mutation, different mechanisms can be predicted such as: (i) loss-of-function for mutations perturbing triple helix formation, intracellular assembly or ColVI secretion; (ii) dominant-negative effect for mutations giving rise to abnormal ColVI polypeptides secreted in the ECM; (iii) haploinsufficiency for mutations affecting mRNA stability, with a decreased synthesis of normal ColVI [90;91;105;106;161;195]. The effect of specific COL6 mutations has to be studied for each clinical case. So far, there is no conclusive evidence on the genotype-phenotype correlation for BM and UCMD, and especially on the relevance of different COL6 mutations for the variable pattern of clinical severity and progression of these two diseases.

7.1. BM

BM (Mendelian Inherited in Man [MIM] 158810) is an autosomal dominantly inherited disease, characterized by a progressive, mild proximal myopathy. It was first described in 1976 by Bethlem and van Wijngaarde [24]. It has been classically identified as a disorder with major impact in adult life, with muscular weakness generally occurring within the first or second decade, although the age of onset is not always easy to identify. Extensor muscles are generally more involved than the flexors, and the proximal muscles more than distal [23]. Magnetic resonance imaging shows a characteristic pattern of involvement of the peripheral muscle region, while the central part tends to be spared. The hallmark of the BM is the presence of contractures of the fingers, wrists, elbows and ankles, as well as hypermobility of distal interphalangeal joints [129] (Figure 13). BM is a very heterogeneous disorder and patients can show mild myopathy as well as more severe signs of a progressive muscular dystrophy. Disability, due to muscular weakness and joint

contractures, is slowly progressive, so that two-thirds of patients over 50 years of age require aids for ambulation [92]. Diaphragm involvement can require respiratory support, while cardiac involvement is usually absent [125]. Patients could also show other clinical features, as keloid formation and follicular hyperkeratosis. This suggests that the altered interaction between ECM and the basement membrane, besides affecting muscles also affects skin. Creatine kinase is normal or only mildly elevated; a myopathic pattern can be detected by electromyography; marked variation in muscle fiber diameter, and the presence of necrotic or dystrophic fibers is detectable in muscle biopsies. Immunohistochemical testing shows normal or mildly reduced levels of ColVI in the endomysium, and quantitative or qualitative ColVI defects can be detected in cultured fibroblasts derived from skin biopsies. Also the reduced expression of laminin b1 has been seen, probably due to a secondary deficiency [130].

7.2. UCMD

UCMD (MIM 254090) is a congenital muscular dystrophy characterized by a severe muscle weakness of early onset, striking hypermobility of distal joints, and proximal joint contractures. In 1930, Ullrich described for the first time this disease as congenital atonic-sclerotic muscular dystrophy. UCMD has been usually identified as an autosomal recessive disorder, even if the model of inheritance is still unclear, and several cases of UCMD with dominant mutations were recently reported [11;143]. Besides, patients with UCMD phenotype but without mutations in the ColVI genes have been described, even if in these cases the new ColVI chains, and related genes, which have been recently discovered [63] could be involved. The clinical signs of UCMD patients, who rarely achieve the ability to walk independently, are a progressive spinal rigidity, scoliosis and hip dislocation (Figure 13). Respiratory failure, unless treated, is a common cause of early death. Skin involvement is similar to that found in BM. Serum creatine kinase activity is usually normal or mildly increased; muscle biopsy reveals a wide spectrum of changes, including variation in fiber size, type I fiber predominance, internal nuclei, focal areas of necrosis and regeneration. Immunohistochemical testing shows a strong reduction or a complete absence of ColVI. Cultured skin fibroblasts of UCMD patients usually show either a markedly decreased secretion of ColVI or lack of the characteristic filamentous network in the ECM, suggesting that UCMD mutations severely affect the synthesis and secretion of ColVI [36;196].



Figure 13. Patients affected by Collagen VI-related disorders.

Typical clinical features of Bethlem myopathy (panels A to E) and of Ullrich congenital muscular dystrophy (panels F and G). From [108].

7.3. The mouse model of ColVI-related disorders

Several years ago Bonaldo *et al.* [26] generated a mutant mouse lacking ColVI with the aim of gaining insight into the function of the ColVI. The *Col6a1*^{-/-} mouse turned out to be the first and to date the only animal model of ColVI-related disorders, and a key to understanding the pathophysiology and devise and test potential therapies for these diseases.

7.3.1. Phenotype

The *col6a1* gene, encoding the $\alpha 1(\text{VI})$ chain, was inactivated by using a targeting vector containing a neomycin resistance cassette in the second exon. The mutation caused the complete absence of the $\alpha 1(\text{VI})$ chain without interfering with the expression of the others; nevertheless, the remaining chains were not able to form a stable triple helical. Thus, the assembly and the secretion of the protein was totally blocked and the homozygous null (*Col6a1*^{-/-}) mice completely lacked ColVI in their tissues. These animals developed normally, were fertile and had no obvious differences with wild type (*wt*). However, a detailed analysis of the tissues showed general signs of myopathy, such as muscle necrosis, regeneration and variability of muscle fiber size. The most affected muscle was the diaphragm, but necrotic fibers were detected also in other skeletal muscles, such as intercostal, and hindlimb muscles. The alterations appeared early (first weeks of age), and presented a limited progression with age. Similar although milder defects were also present in heterozygous (*Col6a1*^{+/-}) mice, indicating functional haploinsufficiency for ColVI [26]. The features displayed by ColVI-deficient mice are thus similar to those detected in patients with ColVI-related disorders. Despite the complete absence of ColVI in the *Col6a1*^{-/-} mice, their phenotype resembles more the milder BM than UCMD [26]. This observation indicates that in the mouse model compensatory mechanisms exist that may limit the net loss of muscle fibers.

7.3.2. Pathogenesis of ColVI-related disorders

Ultrastructural analysis of skeletal muscle from *Col6a1*^{-/-} mice has revealed the presence in about 20–30% of the fibers of marked dilations of the sarcoplasmic reticulum (SR) with normal T-tubules; and of mitochondrial alterations that range from tubular *cristae*, to electron-dense matrix inclusions, to overt swelling [88]. Remarkably, myofibers with mitochondrial-SR alterations also display nuclear features of apoptosis, suggesting (but not proving) a link between organelle changes and increased incidence of cell death. In order to investigate the occurrence of mitochondrial dysfunction in *Col6a1*^{-/-} mice, skeletal muscle fibers from *flexor digitorum brevis* (FDB) were studied. Mitochondrial membrane potential was monitored by fluorescence microscopy using tetramethylrhodamine methyl ester (TMRM), a lipophilic cation that accumulates in energized mitochondria. No differences were observed comparing *Col6a1*^{-/-} with *wt* fibers [88]. Despite the limits posed by comparing fluorescence signals in different cells [21], these findings suggested that the resting potential may not be affected by lack of ColVI [88]. It should be noted, however,

that the experimental procedure of fibers isolation and incubation inevitably selects the best fibers, i.e. those that were either normal or not committed to death at the moment of isolation. After further investigations, a latent mitochondrial dysfunction was shown in *Col6a1*^{-/-} fibers [88].

In normal aerobic conditions the combination of proton pumping by the respiratory chain and of low permeability of the IMM to charged species allows the generation of a high protonmotive force that drives ADP and P_i uptake, and synthesis of ATP by the F₁F₀ ATPase [133]. The earliest consequence of impaired respiration is a switch of the mitochondrial F₁F₀ ATPase from this physiological mode of operation to that of an ATP hydrolase, a condition that occurs when the protonmotive force drops and glycolytic ATP is available. The status of the mitochondrial ATPase can be probed by adding an inhibitor, oligomycin, which should hyperpolarize mitochondria that are synthesizing ATP, and depolarize mitochondria that are using it as fuel instead. Addition of oligomycin caused rapid depolarization in mitochondria from *Col6a1*^{-/-} but not *wt* fibers, an abnormal response that could be prevented by plating FDB fibers on ColVI or by treating them with CsA [88]. The depolarizing effect of oligomycin on mitochondria in *Col6a1*^{-/-} fibers was hypothesized to be due to an increase of PTP flickering in these fibers [18]. Pore flickering causes a slow loss of pyridine nucleotides [48;182] that results in progressive impairment of respiration; and the resulting depolarization causes the switch of the F₁F₀ ATP synthase into an ATP hydrolase. It should be noted that ADP generated by ATP hydrolysis inhibits the PTP; thus, ATP hydrolysis may lead to pore closure and to at least partial restoration of respiration. The maximal attainable rate would be lower than normal, however, and determined by the residual matrix levels of pyridine nucleotides. As long as the energy demand can be matched the fiber would behave normally, but dysfunction would be precipitated by increased workload and/or by increased PTP flickering, events that would eventually lead to individual muscle fiber death as observed in the *Col6a1*^{-/-} mice. Moreover, further studies performed in patients affected by UCMD have suggested that the mechanism that causes depolarization after oligomycin could involve also Ca²⁺ deregulation and the sarcoplasmic reticulum [4] (see paragraph 7.3.3). *Col6a1*^{-/-} myofibers show a higher incidence of apoptosis compared to *wt* cells, and addition of oligomycin causes a dramatic increase of apoptotic nuclei which is completely normalized by treatment with CsA. The therapeutic efficacy of *in vivo* CsA administration in the *Col6a1*^{-/-} mice suggests that PTP plays a key role in the pathogenesis of the mouse model of this disease. As already mentioned, however, CsA could influence mitochondrial morphology and function through its calcineurin-dependent inhibition of Drp1 phosphorylation [37]; and calcineurin has been shown to affect skeletal muscle physiology by itself [12;145], so further investigations appear to be necessary to evaluate the role of PTP in these model. Finally, the connection between the molecular lesion of a component of the ECM due to a genetic disease and the induction of a mitochondria-dependent apoptosis in muscle cells raises the question of how do mitochondria sense the events occurring in the extracellular space. It is possible that normal interactions involving integrins and intracellular signaling pathways could be altered when ECM is lacking ColVI; this could result in mitochondrial ROS production [187], or in a modification of the expression levels of Bcl-2-related

proteins [159]. A further mechanism, involving laminin, dystrophin and the actin cytoskeleton could also be considered, given that the fusion mechanism and the regulation of mitochondrial distribution are under the control of cytoskeletal motors and small GTPase [124]. Moreover, mitochondrial fusion/fission mechanisms are involved in the control of apoptosis [149] and the integrity of the mitochondrial net is essential for muscle cell metabolism [170].

7.3.3. From mouse to man

In order to establish whether latent mitochondrial dysfunction and increased incidence of apoptosis are involved in the pathogenesis of the genetically and clinically heterogeneous UCMD and to assess whether they can be reverted by CsA, biopsies and primary muscle cell cultures from UCMD patients were studied [5]. The patients included in the study were representative of the spectrum of severity of UCMD evaluated by determining the expression of ColVI and the ability to stand and walk; and mutations affected both COL6A1 and COL6A3 genes. As seen in the *Col6a1*^{-/-} mice, the treatment of cultures from UCMD patients with CsA prevented mitochondrial dysfunction and normalized the incidence of apoptosis, suggesting that inappropriate opening of the PTP could be the basis for both events [5]. It was also interesting that the mitochondrial defect was revealed irrespective of the genotypic profile of ColVI genes (homozygous or heterozygous mutations) and of the ColVI expression in biopsies. Therefore, additional environmental factors may play a role in the individual susceptibility to muscle fiber demise and regeneration. Another study performed in muscle cell culture from UCMD patients has demonstrated that also rotenone (inhibitor of the complex I of the mitochondrial respiratory chain) causes mitochondrial depolarization, while this does not happen in cells from a healthy donor [4]. The current hypothesis to explain this event is that in healthy cells rotenone causes a decrease of membrane potential that is readily compensated by reversal of the ATP synthase, thus preventing substantial depolarization. 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 cells, the PTP threshold is instead very close to the resting membrane potential (as a result of increased matrix Ca²⁺ levels and/or of PTP sensitization to other factors), a condition that causes PTP opening even for small depolarizations. Because of the increased inner membrane permeability repolarization cannot occur despite ATP hydrolysis, which indeed proceeds at a faster rate. CsA normalized the response to rotenone moving the threshold away from the resting potential and restoring the normal response to rotenone (Figure 14)[4].

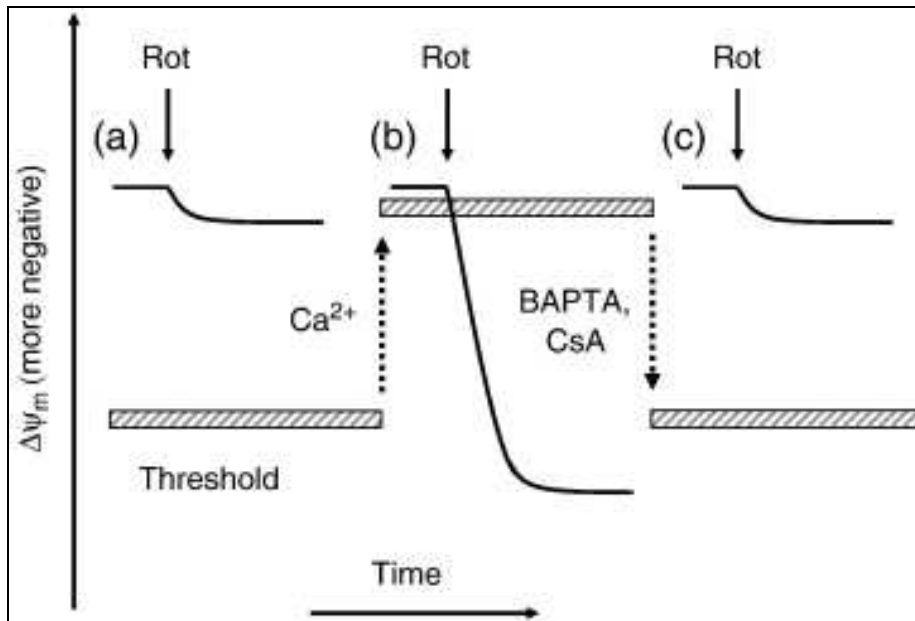


Figure 14. Shift of PTP voltage threshold in UCMD.

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. From [4].

Depolarization after addition of oligomycin could be due to an indirect effect on the pore mediated by a shift of the voltage threshold due to the Ca^{2+} overload, an event where dysfunction of the SR could be critical (Figure 15). 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 [93;165]. 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+} concentration [30;94]. Addition of oligomycin causes inhibition of mitochondrial ATP synthesis, which is followed by a rapid drop of total cellular ATP only in the UCMD cells. This will cause decreased Ca^{2+} uptake by the SR and decreased Ca^{2+} extrusion by the plasma membrane Ca^{2+} pumps. The result is 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. It is noteworthy that oligomycin-induced mitochondrial depolarization is often preceded by a lag phase [4;5], which may well represent the time required for the shift of the threshold. Moreover, depolarization is prevented by intracellular Ca^{2+} chelators [4], and 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.

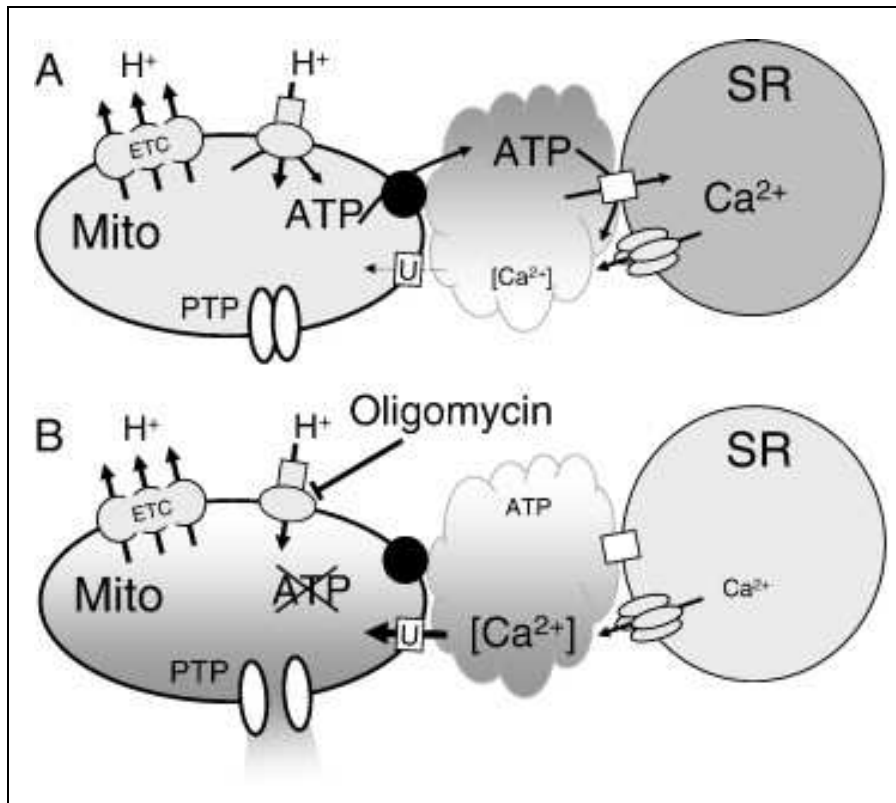


Figure 15. Mitochondria-SR interactions in regulation of ATP production and Ca²⁺ homeostasis. Effect of oligomycin.

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 channeled to the SR where it fuels the Ca²⁺ pumps. Ca²⁺ release at the mouth of the release channels is relayed to the matrix through the Ca²⁺ uniporter (U) to regulate ATP synthesis. Panel B, inhibition of the ATP synthase by oligomycin decreases the ATP available to the Ca²⁺ pumps and causes a shift of Ca²⁺ from the SR to mitochondria, eventually resulting in PTP opening that is particularly likely to occur in UCMD. From [4]

Although the mechanisms that regulate the pathogenesis of the ColVI-related disorders are still obscure, encouraging results were obtained with an open pilot trial of UCMD and BM patients [126]. Five patients representative of the clinical and molecular variety of BM and UCMD were treated with CsA for 1 month. Apoptosis, mitochondrial dysfunction, and muscle regeneration were evaluated before and after the pharmacological treatment. The results showed that CsA treatment normalized the mitochondrial membrane potential response to oligomycin; dramatically decreased the apoptotic rate of muscle fibers; and significantly increased muscle regeneration, an effect that was prominent in younger patients [126]. Thus, the treatment with moderate doses of CsA is effective on patients affected by ColVI-related disorders. As already mentioned, however, the potential benefit of treatment with CsA must be carefully weighed against the potential increase of infections that may follow immunosuppression.

RESULTS

I.

The aim of this work was to clarify whether the beneficial effects of CsA in ColVI-related disorders depend uniquely on PTP desensitization through its inhibition of CyP-D, or on the action of this drug on additional targets.

The results are reported in the publication: Palma E, Tiepolo T, Angelin A, Sabatelli P, Maraldi NM, Basso E, Forte MA, Bernardi P, Bonaldo P (2009) Genetic ablation of cyclophilin D rescues mitochondrial defects and prevents muscle apoptosis in collagen VI myopathic mice, *Hum Mol Genet* 18:2024-31, which is enclosed at pp. 38-50.

Genetic ablation of cyclophilin D rescues mitochondrial defects and prevents muscle apoptosis in collagen VI myopathic mice

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Ullrich congenital muscular dystrophy (UCMD) and Bethlem myopathy are inherited muscle disorders caused by mutations of genes encoding the extracellular matrix protein collagen VI (ColVI). Mice lacking ColVI (*Col6a1*^{-/-}) display a myopathic phenotype associated with ultrastructural alterations of mitochondria and sarcoplasmic reticulum, mitochondrial dysfunction with abnormal opening of the permeability transition pore (PTP) and increased apoptosis of muscle fibers. Treatment with cyclosporin (Cs) A, a drug that desensitizes the PTP by binding to cyclophilin (Cyp)-D, was shown to rescue myofiber alterations in *Col6a1*^{-/-} mice and in UCMD patients, suggesting a correlation between PTP opening and pathogenesis of ColVI muscular dystrophies. Here, we show that inactivation of the gene encoding for Cyp-D rescues the disease phenotype of ColVI deficiency. In the absence of Cyp-D, *Col6a1*^{-/-} mice show negligible myofiber degeneration, rescue from mitochondrial dysfunction and ultrastructural defects, and normalized incidence of apoptosis. These findings (i) demonstrate that lack of Cyp-D is equivalent to its inhibition with CsA at curing the mouse dystrophic phenotype; (ii) establish a cause–effect relationship between Cyp-D-dependent PTP regulation and pathogenesis of the ColVI muscular dystrophy and (iii) validate Cyp-D and the PTP as pharmacological targets for the therapy of human ColVI myopathies.

INTRODUCTION

Collagen VI (ColVI) is a broadly distributed extracellular matrix protein forming a microfilamentous network that is particularly abundant in skeletal muscle (1,2). The protein is composed of three distinct α -chains encoded by separate genes (*COL6A1*, *COL6A2* and *COL6A3* in humans). ColVI is synthesized and secreted by cells organizing an extracellular matrix, and transcriptional regulation is a key step in its production. In muscle, ColVI is a major component of the endomysium, where it is localized just outside the basement membrane of muscle fibers (3,4). Deficiency of ColVI in humans, due to mutations in *COL6A1–A3* genes, is one of the most common causes of congenital muscular dystrophies (1). Two main syndromes were described, Bethlem myopathy

(MIM#158810) and Ullrich congenital muscular dystrophy (UCMD) (MIM#254090), but additional muscle phenotypes were recently linked to ColVI defects (5). Bethlem myopathy is a relatively mild and slowly progressive myopathic disorder, whereas UCMD is a severe and rapidly progressive muscle disease usually causing early death due to respiratory failure.

Studies with *in vitro* cell cultures derived from skeletal muscles have demonstrated that ColVI is largely produced by the interstitial fibroblasts and not by the myogenic cell population (4). ColVI secreted by muscle interstitial fibroblasts was found to adhere to the surface of myotubes and establish a dense pericellular matrix around myogenic cells (4). Interestingly, a recent study has shown that transcription of the *Col6a1* gene by muscle interstitial fibroblasts is strictly

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dependent on a specific enhancer region, whose activation requires inductive signals from myogenic cells (6). These findings suggest that synergistic activities between these two cell types are crucial in regulating ColVI synthesis and function in muscle. Three novel genes (*COL6A4–A6*) coding for additional ColVI subunits and showing tissue-regulated expression were recently identified (7), thus increasing the spectrum of potential primary structures for ColVI.

A substantial contribution to understanding the pathogenesis of ColVI diseases was provided by mice lacking ColVI obtained by means of targeted inactivation of *Col6a1* gene. We have shown that *Col6a1*^{-/-} mice have an early onset myopathic phenotype affecting diaphragm and other skeletal muscles, and display spontaneous apoptosis and ultrastructural defects of mitochondria and sarcoplasmic reticulum (8,9). Muscle fibers derived from *Col6a1*^{-/-} mice have a latent mitochondrial dysfunction due to abnormal opening of the permeability transition pore (PTP) (9), a high-conductance channel located in the mitochondrial inner membrane that plays a role in several forms of cell death (10). Consistently, treatment of *Col6a1*^{-/-} mice with cyclosporin A (CsA), a widely used immunosuppressant that desensitizes the mitochondrial PTP independent of calcineurin inhibition (11), led to a marked decrease of muscle fiber apoptosis and of ultrastructural lesions (9). Guided by these findings, we showed that patients affected by UCMD have strikingly similar abnormalities, which could be normalized by treatment with CsA or its non-immunosuppressive derivative Debio 025 (12,13). These observations indicate that the muscle lesions caused by ColVI deficiency can be largely reverted by desensitizing the mitochondrial PTP, yet the mechanistic proof that a cause–effect relationship between PTP opening and the myopathy exists is still lacking.

The mitochondrial target for CsA is cyclophilin (CyP)-D, a peptidyl-prolyl *cis–trans* isomerase located in the mitochondrial matrix that is inhibited by CsA in the same range of concentrations desensitizing the PTP (11,14). Oxidative and other cellular stresses promote the recruitment of CyP-D to the mitochondrial inner membrane where it favours opening of the PTP (10,15). Mice with targeted inactivation of *Ppif*, the gene coding for CyP-D, were produced and characterized by different laboratories (16–19). Mitochondria isolated from *Ppif*^{-/-} mice show a marked resistance to PTP opening, with a significant desensitization of the PTP to Ca²⁺ overload and lack of further desensitizing effects by CsA (17).

Here, we show that genetic ablation of CyP-D markedly attenuates the phenotypic defects of ColVI-deficient mice. These findings are an important proof of principle that abnormal opening of mitochondrial PTP plays a key mechanistic role in the pathogenesis of ColVI myopathies.

RESULTS

Lack of CyP-D ameliorates muscle histology of *Col6a1* knockout mice

Breeding of *Col6a1*^{-/-} and *Ppif*^{-/-} mice allowed the generation of animals with either wild-type (WT) (*Col6a1*^{+/+}*Ppif*^{+/+}), ColVI null (*Col6a1*^{-/-}*Ppif*^{+/+}), CyP-D null

(*Col6a1*^{+/+}*Ppif*^{-/-}) or ColVI/CyP-D double null (*Col6a1*^{-/-}*Ppif*^{-/-}) genotypes. As reported previously for *Ppif*^{-/-} mice (16–19), *Col6a1*^{+/+}*Ppif*^{-/-} individuals did not display an overt phenotype, and we verified the lack of muscle abnormalities through histological analysis, electron microscopy and detection of apoptosis (results not shown). *Col6a1*^{-/-}*Ppif*^{-/-} mice were born at the expected Mendelian ratios, they were fertile and did not show any overt phenotypic abnormality.

We first performed a histological examination of skeletal muscles from WT, ColVI null and ColVI/CyP-D double null mice. In agreement with previous observations, *Col6a1*^{-/-} muscles showed typical signs of myopathy, with focal areas of infiltration by phagocytic cells and a pronounced variation of fiber diameter (Fig. 1Ab and e). These pathological signs were markedly reduced in *Col6a1*^{-/-}*Ppif*^{-/-} muscles, which showed more uniform fiber size and decreased tissue inflammation (Fig. 1Ac and f). Morphometric analysis of myofiber cross-sectional areas in tibialis anterior muscle confirmed that *Col6a1*^{-/-} contained myofibers of different sizes, with a significant incidence of small fibers, whereas both WT and *Col6a1*^{-/-}*Ppif*^{-/-} muscles contained almost exclusively large fibers (Fig. 1B). Although the amount of myofibers with central nuclei was not very high in ColVI null mice (8,9), *Col6a1*^{-/-} muscles showed about 8-fold higher incidence of fibers with centrally located nuclei (WT: 0.38 ± 0.16; *Col6a1*^{-/-}: 3.01 ± 0.71), and this percentage was decreased by half in *Col6a1*^{-/-}*Ppif*^{-/-} muscles (1.56 ± 0.03).

We investigated in further detail the histological structure of diaphragm after systemic injection with Evans blue dye (EBD), a small molecular tracer that only permeates the sarcolemma of damaged fibers. Both visual inspection and microscopic analysis showed that WT animals excluded the dye (Fig. 1Ca and a'), that marked uptake of EBD took place in several fibers in the diaphragm of *Col6a1*^{-/-} mice (Fig. 1Cb and b') and that the number of fibers taking up the dye was remarkably low in the diaphragm of *Col6a1*^{-/-}*Ppif*^{-/-} mice (Fig. 1Cc and c'). Detailed microscopic quantification of EBD-positive fibers in diaphragm transverse sections revealed that 14.6 ± 0.7% of *Col6a1*^{-/-} muscle fibers were permeable to the tracer, a figure that was reduced to 1.2 ± 0.2% in *Col6a1*^{-/-}*Ppif*^{-/-} mice, with a 12-fold decrease compared with *Col6a1*^{-/-} animals (Fig. 1D). These data suggest that ablation of CyP-D largely prevents the myopathic defects of ColVI-deficient mice.

CyP-D ablation attenuates ultrastructural defects of ColVI-deficient muscle fibers

Electron microscopic analysis confirmed the characteristic ultrastructural alterations of *Col6a1*^{-/-} (9) that are never observed in diaphragms from WT individuals (Fig. 2A). *Col6a1*^{-/-} muscle fibers displayed mitochondrial defects with swelling, hypodense matrix and abnormal cristae, and dilations of sarcoplasmic reticulum (Fig. 2Ab and b'). In *Col6a1*^{-/-}*Ppif*^{-/-} mice, lack of CyP-D markedly decreased ultrastructural alterations in diaphragm muscle fibers (Fig. 2Ac and c'). Analysis of about 300 fibers from three different animals of each genotype showed that *Col6a1*^{-/-}

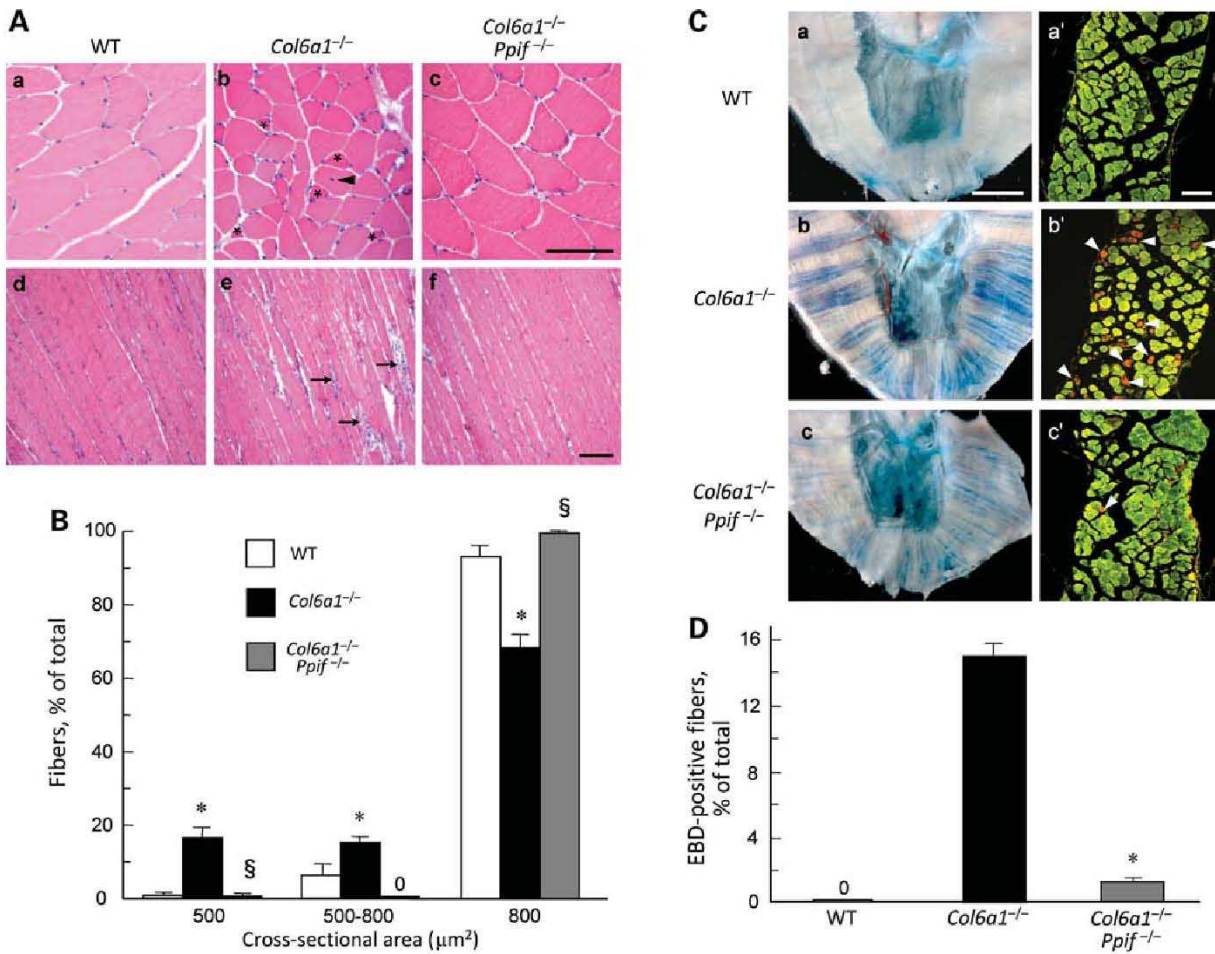


Figure 1. Histological analysis of skeletal muscles from WT, KO (*Col6a1*^{-/-}) and DKO (*Col6a1*^{-/-}*Ppif*^{-/-}) mice. (A) Representative cross (a–c) and longitudinal sections (d–f) of H&E staining of tibialis anterior muscle from 24-week-old WT (a and d), *Col6a1*^{-/-} (b and e) and *Col6a1*^{-/-}*Ppif*^{-/-} (c and f) mice. *Col6a1*^{-/-} muscles show a large variability in myofiber size, with several fibers of very small diameter (asterisk), presence of regenerating fibers with centrally located nuclei (arrowhead) and occurrence of inflammatory infiltrates (arrows). On the contrary, *Col6a1*^{-/-}*Ppif*^{-/-} muscles display uniform myofiber size and absence of inflammatory infiltrates, similarly to WT muscles. Scale bar, 100 μm . (B) Morphometric analysis of myofiber cross-sectional areas in tibialis anterior muscles of the different genotypes. Fibers were grouped into three size ranges, and at least 500 fibers of each genotype were analysed using LEICA IM 1000 software (Leica Microsystems). Data are expressed as mean \pm SEM. **P* < 0.05 versus WT; [§]not significant (*P* = 0.64) versus WT. (C) EBD uptake in the diaphragm of WT, *Col6a1*^{-/-} and *Col6a1*^{-/-}*Ppif*^{-/-} mice. Following injection with EBD, diaphragms were isolated from WT (a and a'), KO (b and b') and DKO (c and c') mice, and examined by light microscopy (a–c). (a'–c') Representative cross-sections visualized by fluorescence microscopy, where myofibers penetrated by EBD appear red (arrowheads). Scale bars, 500 μm (a–c) and 100 μm (a'–c'). (D) Quantification of EBD-positive fibers in diaphragms of different genotypes. At least five animals of each genotype were analysed, and quantification was carried out in 20 randomly chosen cross-sections covering different areas of the diaphragm. Data are expressed as mean \pm SEM. **P* < 0.05 versus *Col6a1*^{-/-}.

Ppif^{-/-} mice had a 3-fold lower incidence of fibers with abnormal mitochondria than *Col6a1*^{-/-} individuals and that this incidence was in the same range of that observed in WT animals (Fig. 2B). Mitochondria of *Col6a1*^{-/-}*Ppif*^{-/-} myofibers typically displayed tightly packed cristae, with a structural organization indistinguishable from that of WT fibers (Fig. 2A, compare panels a' and c'). Dilations of sarcoplasmic reticulum were still found in *Col6a1*^{-/-}*Ppif*^{-/-} muscles (Fig. 2Ac), but the incidence of fibers with these alterations was lower than that observed in *Col6a1*^{-/-} muscles, decreasing from 28.1 ± 4.8 to $10.5 \pm 5.0\%$ (*P* < 0.005).

Lack of CyP-D prevents mitochondrial depolarization in myofibers and cultured muscle cells of ColVI-deficient mice

We next assessed the mitochondrial membrane potential by monitoring the accumulation of tetramethylrhodamine methyl ester (TMRM) in *flexor digitorum brevis* (FDB) muscle fibers derived from mice with the different genotypes. We previously demonstrated that *Col6a1*^{-/-} myofibers display an anomalous depolarizing response elicited by the addition of oligomycin, an inhibitor of the mitochondrial F1FO ATPase (9). As expected, the addition of oligomycin

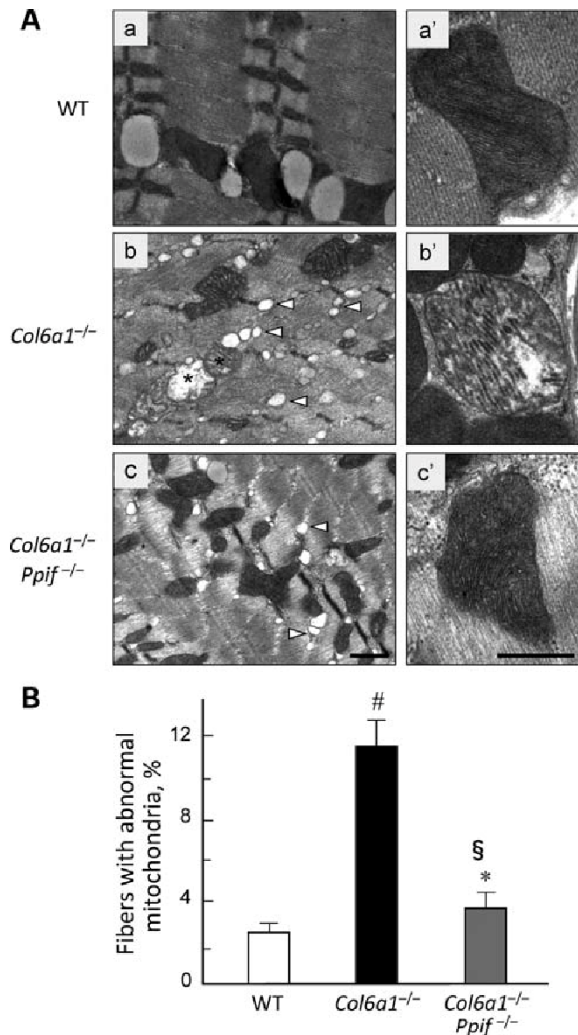


Figure 2. (A) Electron micrographs of diaphragm of WT (a and a'), *Col6a1*^{-/-} (b and b') and *Col6a1*^{-/-}*Ppif*^{-/-} (c and c') mice. The pictures show representative fields of whole diaphragm sections (a–c) and mitochondria (a'–c'). In (a–c), enlarged sarcoplasmic reticulum and swollen mitochondria are labelled by arrowheads and asterisks, respectively. Scale bars, 400 nm. (B) Frequency of fibers with swollen mitochondria. Data represent the mean of at least three independent experiments ± SD. #*P* < 0.05 versus WT; **P* < 0.05 versus *Col6a1*^{-/-}; §not significant (*P* = 0.12) versus WT.

caused higher incidence of fibers with depolarizing mitochondria in *Col6a1*^{-/-} compared with WT mice, the fraction of fibers with depolarizing mitochondria after oligomycin addition being, respectively, 33 and 10% for these two genotypes (Fig. 3A, left and middle panels). In *Col6a1*^{-/-}*Ppif*^{-/-} individuals, lack of CyP-D led to an increased resistance to oligomycin-induced mitochondrial depolarization, and the fraction of fibers with depolarizing mitochondria dropped to 14% (Fig. 3A, right panel). Our previous studies on isolated myofibers and primary muscle cell cultures indicated that the deficiency of extracellular ColVI results in an increased open propensity of the PTP, thus impinging on the

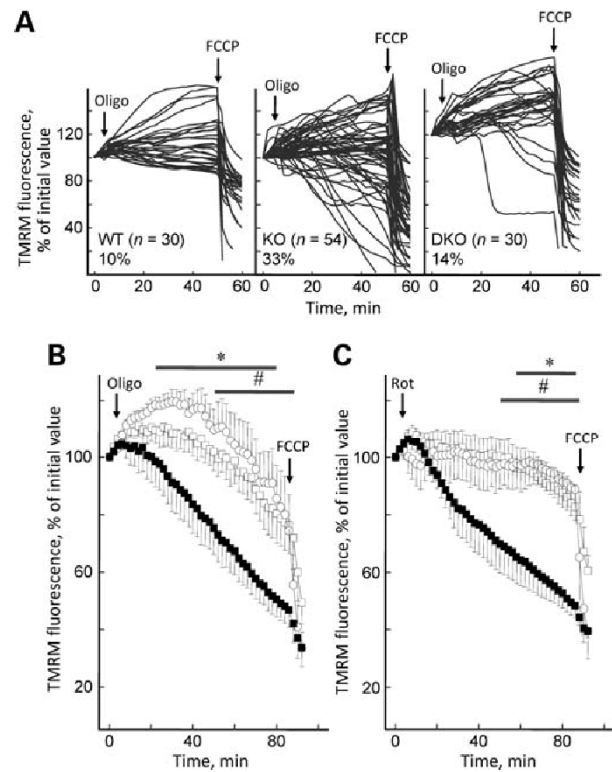


Figure 3. (A) Mitochondrial response to oligomycin in muscle fibers of WT (*n* = 30), *Col6a1*^{-/-} (KO, *n* = 54) and *Col6a1*^{-/-}*Ppif*^{-/-} (DKO, *n* = 30) mice. Myofibers were isolated from FDB muscle and loaded with TMRM (20 nM) for 15 min at 37°C. Where indicated (arrows), oligomycin (Oligo, 5 μM) or the protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP, 4 μM) were added. The probe accumulates in mitochondria that maintain the membrane potential. Results of at least three experiments per genotype are shown, and each trace represents the fluorescence values of a single fiber. The fraction of depolarizing fibers (as per cent of total) is also provided for each genotype, where fibers are considered as depolarizing when they lose more than 10% of initial value of TMRM fluorescence after oligomycin addition. (B and C) Changes of mitochondrial TMRM fluorescence induced by oligomycin (B) or rotenone (C) in primary muscle cell cultures derived from diaphragms. Cells from WT (open circles), *Col6a1*^{-/-} (closed squares) and *Col6a1*^{-/-}*Ppif*^{-/-} (open squares) were loaded with 10 nM TMRM for 30 min at 37°C. Where indicated, oligomycin (Oligo, 5 μM), rotenone (Rot, 2 μM) or carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP, 4 μM) were added. Data represent the mean of at least four independent experiments ± SEM. The upper bars indicate all the time points where *P* < 0.05 for *Col6a1*^{-/-} compared with either WT (*) or with *Col6a1*^{-/-}*Ppif*^{-/-} (#).

mitochondrial ability to maintain the membrane potential in response to various treatments (9,12,20). Based on these observations, we monitored TMRM fluorescence in primary diaphragm cell cultures derived from mice with the three genotypes, in order to assess how ablation of CyP-D affected mitochondrial membrane potential in different experimental conditions. A marked decrease of TMRM fluorescence was induced by either oligomycin or rotenone in primary muscle cultures from *Col6a1*^{-/-} mice, but not in those from WT and *Col6a1*^{-/-}*Ppif*^{-/-} animals (Fig. 3B and C). Addition of the protonophore carbonylcyanide-*p*-trifluoromethoxyphenyl hydrazone promptly collapsed fluorescence in all cultures,

thus confirming that the observed variations in TMRM fluorescence were indeed measuring the mitochondrial membrane potential. The responses elicited by oligomycin and rotenone in WT and *Col6a1*^{-/-} *Ppif*^{-/-} cells were remarkably similar, and both cultures were able to maintain high TMRM fluorescence after more than 1 h of incubation in the presence of either inhibitor (Fig. 3B and C).

CyP-D inactivation prevents muscle apoptosis in ColVI-deficient mice

We finally investigated whether genetic ablation of CyP-D was able to improve cell survival and to counteract the apoptotic phenotype observed in *Col6a1*^{-/-} mice (9). The frequency of apoptotic nuclei was evaluated *in situ* by using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) method. TUNEL-positive nuclei were readily detected in the diaphragm of *Col6a1*^{-/-} mice, but not in the corresponding samples of WT and *Col6a1*^{-/-} *Ppif*^{-/-} mice (Fig. 4A). *Col6a1*^{-/-} muscle sections displayed an average of 57.0 ± 3.8 TUNEL-positive nuclei/mm², while the incidence in *Col6a1*^{-/-} *Ppif*^{-/-} sections was 4.8 ± 1.6, which is not significantly different from the value (2.0 ± 1.2) measured in WT sections (Fig. 4B). To investigate further the link between CyP-D ablation and increased cell survival, we also assessed the occurrence of apoptosis in cultured muscle cells. Again, cultures from *Col6a1*^{-/-} *Ppif*^{-/-} and WT muscles displayed similar frequencies of TUNEL-positive nuclei, and these were significantly lower than those displayed by cultures from *Col6a1*^{-/-} muscles (Fig. 4C). Growth of *Col6a1*^{-/-} *Ppif*^{-/-} cultures in serum-free medium caused a 5-fold increase in the incidence of TUNEL-positive nuclei; as expected of cells lacking CyP-D, however, the apoptotic response to serum starvation was not prevented by treatment with CsA (Fig. 4C).

DISCUSSION

The results of the present manuscript provide compelling evidence for the mitochondrial pathogenesis of ColVI myopathies, which we had previously suggested based on ultrastructural, functional and pharmacological data (9,12,13,20). Mitochondrial dysfunction can be readily demonstrated in skeletal muscle fibers and muscle-derived cultures both from *Col6a1*^{-/-} mice and from patients affected by UCMD (9,12,13,20), as also recently confirmed by another laboratory (21). These findings, however, as well as the striking normalizing effect of CsA on mitochondrial function (9,12,13,20,21) and apoptosis (9,12,13,20) provided an intriguing correlation but not the proof that a cause-effect relationship exists. Indeed, two major issues should be considered when interpreting the pharmacological effects of CsA on ColVI myopathies.

CsA also inhibits calcineurin, which has been shown to affect skeletal muscle physiology (22) and mitochondrial fission through Drp-1 dephosphorylation (23). We had previously addressed this issue in the *Col6a1*^{-/-} mouse and found that inhibition of calcineurin with FK506, which does not inhibit CyPs and has no effects on the PTP (24), could

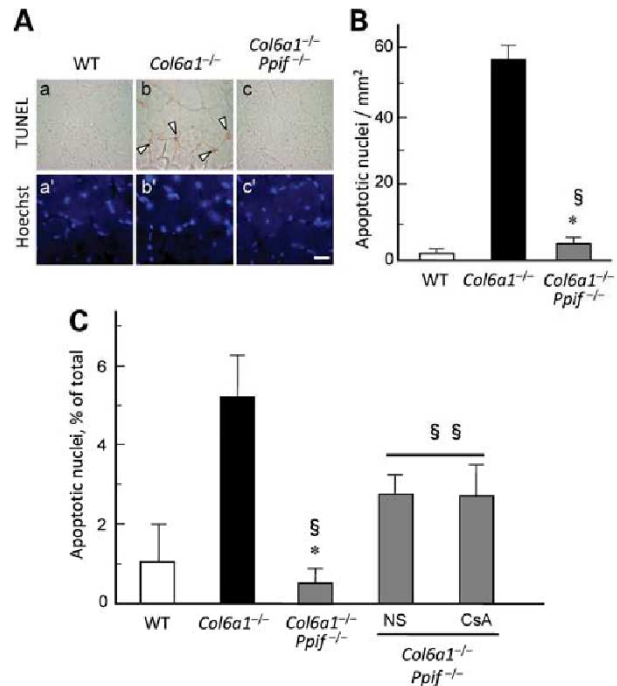


Figure 4. Incidence of apoptosis in muscle (A and B) and in cell cultures (C) derived from WT, *Col6a1*^{-/-} and *Col6a1*^{-/-} *Ppif*^{-/-} mice. (A) TUNEL staining of diaphragms. Hoechst-stained sections are shown below the corresponding TUNEL pictures. Several TUNEL-positive nuclei (arrowheads) are present in KO, but not in WT and DKO muscles. Scale bar, 50 μm. (B) Incidence of TUNEL-positive nuclei in diaphragm muscle fibers with the indicated genotype. Data are expressed as mean ± SEM of at least three independent experiments. For each genotype, 20–30 animals were analysed. **P* < 0.05 versus *Col6a1*^{-/-}; §not significant (*P* = 0.13) versus WT. (C) Primary muscle cells were derived from diaphragms of mice with the stated genotypes, cultured on plastic dishes and scored for the presence of TUNEL-positive nuclei. Where indicated, cells were maintained for 2 h in serum-free (NS) medium in the absence or presence of 1.6 μM CsA. Data are the mean of four independent experiments ± SEM. **P* < 0.05 versus *Col6a1*^{-/-}; §not significant (*P* = 0.41) versus WT; §§not significant (*P* = 0.93).

not reproduce the protective effects of CsA but rather worsened the incidence of apoptosis (9). However, it is difficult to extrapolate the effects obtained in the mouse model to human ColVI diseases, which are genetically and functionally so heterogeneous (1). A strong indication that calcineurin is not involved in the effects of CsA in the patients was obtained through the use of D-MeAla³-EtVal⁴-cyclosporin (Debio 025), a CyP inhibitor that does not affect calcineurin. Indeed, Debio 025 normalized the mitochondrial phenotype and decreased the incidence of apoptosis in cultures from UCMD patients (12) and *Col6a1*^{-/-} mice (44); yet it was 7000 times less active than CsA at inhibiting interleukin-2 production in Jurkat cells and at least 15 times less potent in mixed lymphocyte reaction tests (25).

Even if a role for calcineurin inhibition in the protective effects of CsA could be ruled out in ColVI deficiencies, a second problem is that CsA (and Debio 025) binds to and inhibits all members of the CyP family of peptidyl-prolyl *cis*-*trans* isomerases (26), which in humans includes

17 unique proteins (27). Besides their possible implication in ColVI myopathies, CyPs play important roles in a number of human diseases that include inflammation and vascular dysfunction (28–32), wound healing (33), innate immunity to HIV (34), hepatitis C infection (35), host–parasite interactions (36), tumour biology (37) and several pathological conditions mediated by the mitochondrial PTP through matrix CyP-D (10). Thus, and despite the demonstrable beneficial effects of CsA and Debio 025 on mitochondria in ColVI diseases (9,12,13,20), it is impossible to sort the consequences of CyP-D inhibition from those caused by inhibition of other CyPs, which might independently affect muscle fiber apoptosis. Thus, assessing whether a cause–effect relationship exists between PTP inhibition and therapy of ColVI muscular dystrophies remained a major challenge that could only be addressed by genetics.

In the present study, we took advantage of a CyP-D null mouse strain that we had developed and characterized previously (17,38). Mitochondria isolated from these mice display a decreased sensitivity of the PTP to opening that matches quite precisely the effects of CsA in WT individuals (17) provided that inorganic phosphate is present (38). Despite lack of an overt phenotype, CyP-D null mice proved to be extremely resistant to ischaemia-reperfusion injury of the heart (16,18) and brain (19). Crossing of these mice with the *Col6a1*^{-/-} mouse (8) offered, therefore, a unique opportunity to test whether genetic ablation of CyP-D could mimic the protective effects of CsA on the myopathy.

The striking result of the present work is that *Col6a1*^{-/-} *Ppif*^{-/-} mice no longer display the disease phenotype of *Col6a1*^{-/-} individuals, which is characterized by histological features of myopathy, permeability of skeletal muscle fibers to small tracers (8), ultrastructural lesions of the mitochondria and sarcoplasmic reticulum (9), latent mitochondrial dysfunction that can be triggered by oligomycin (9) or rotenone (20) and increased rates of apoptosis (9). With the exception of a small, residual dilation of the sarcoplasmic reticulum, all these hallmarks of the ColVI disease disappeared in *Col6a1*^{-/-} *Ppif*^{-/-} mice. This set of findings demonstrates that genetic ablation of CyP-D, a molecule that favours PTP opening by shielding it from the protective effects of inorganic phosphate (38), has the same beneficial effects on the myopathic phenotype caused by ColVI deficiency as pharmacological inhibition of CyP-D with CsA or Debio 025 (9,12,13,20). Our findings match recent genetic and pharmacological studies that extend the role of the PTP to mouse models of Duchenne muscular dystrophy (39,40) and MDC1A (39).

Taken together, these results have tremendous implications for the therapeutic perspectives of muscular dystrophies, in general, and of ColVI diseases, in particular. UCMD is a chronic muscle wasting disease involving the diaphragm, and respiratory failure is a common complication which is worsened by pulmonary infections (41). Long-term treatment with CsA exposes the patients to the untoward effects of immunosuppression, which may favour life-threatening infections. The present identification of the PTP as a causative event in muscle cell demise in ColVI muscular dystrophy and the genetic validation of CyP-D as a calcineurin-independent pharmacological target represent a fundamental step

towards a therapy of human ColVI muscular dystrophies with CyP inhibitors.

MATERIAL AND METHODS

Mice

Col6a1^{-/-} mice were crossed with *Ppif*^{-/-} mice. Both strains were previously characterized and were in the inbred C57BL/6 background (8,17). All experiments were performed by comparing sex matched, 8- to 24-week-old animals with either *Col6a1*^{-/-} *Ppif*^{+/+} [ColVI knockout (KO)], *Col6a1*^{-/-} *Ppif*^{-/-} [ColVI/CyP-D double knockout (DKO)] or *Col6a1*^{+/+} *Ppif*^{+/+} (WT) genotypes. Mouse procedures were approved by the competent Authority of the University of Padova and authorized by the Italian Ministry of Health.

Histology and EBD uptake

Tibialis anterior muscles were removed, frozen in isopentane, cooled in liquid nitrogen and kept at -80°C until use. Longitudinal and cross-sections ($7\ \mu\text{m}$) were prepared and processed for haematoxylin and eosin (H&E) staining. EBD staining of muscles *in vivo* was performed by i.p. injection with 0.2 ml EBD (10 mg/ml in phosphate-buffered saline, Sigma). Mice were sacrificed after 16–18 h and diaphragms were fixed with 4% paraformaldehyde overnight at 4°C , dehydrated in a graded series of water/ethanol mixtures and embedded in paraffin. Seven-micrometre-thick sections were cut and examined with a Zeiss Axioplan fluorescence microscope (20 \times magnification) to locate EBD distribution after the removal of paraffin and mounting in Permount.

Electron microscopy

Diaphragm muscles were isolated from mice, gently stretched on wax, fixed with 2.5% glutaraldehyde in phosphate buffer 0.1 M (pH 7.4), postfixed with 1% osmium tetroxide in veronal buffer and embedded in Epon E812 resin. Ultrathin sections were stained with uranyl acetate and lead citrate, and observed in a Philips EM400 transmission electron microscope at 100 kV. For statistical analysis, 300 myofibers were observed from three different tissue blocks for each sample. Data were analysed with the Mann–Whitney test, and values with $P < 0.05$ were considered significant.

Isolation of skeletal muscle fibers

Fibers were isolated from FDB muscles as previously described (9,42). Intact myofibers were plated onto 24 mm round glass coverslips coated with laminin ($3\ \mu\text{g cm}^{-2}$) and cultured for 24 h in Dulbecco's modified Eagle medium (DMEM, Sigma) containing 10% fetal calf serum (FCS, Sigma), before starting the experiment.

Primary muscle cell cultures

Cultures enriched in myoblasts were prepared by enzymatic and mechanical dissociation of diaphragms from 8-week-old mice, using a protocol modified from Rando and Blau (43).

Briefly, the dissected muscles were minced and incubated for 40 min at 37°C in DMEM supplemented with 0.5% collagenase type I (Sigma). Cells released from the tissue were passed through a 80 µm filter, collected by centrifugation and resuspended in DMEM supplemented with 20% FCS. The cell suspension was plated in culture dishes and incubated at 37°C for 15 min, to allow for adhesion of fibroblasts. Floating cells, consisting of an enriched myoblast population, were transferred to gelatin-coated dishes and grown at 37°C and 5% CO₂ in DMEM supplemented with 20% FCS. Cultures were characterized as described in the supplemental material (Supplementary Material, Figs S1 and S2; and Table S1), and the relative proportion of myoblasts versus fibroblasts was carefully determined. Primary cultures within passage 6 were used for all experiments.

TMRM assay

Mitochondrial membrane potential was measured by epifluorescence microscopy based on the accumulation of TMRM fluorescence. FDB myofibers were placed in 1 ml Tyrode's buffer and loaded with 20 nM TMRM (Molecular Probes) as previously described (9). Primary muscle cells cultures were seeded onto 24 mm-diameter round glass coverslips, grown for 2 days in DMEM supplemented with 20% FCS and studied as described after loading with 10 nM TMRM in 1 ml of serum-free DMEM (12).

TUNEL assay

Paraffin sections (7 µm thick) were prepared from diaphragm muscles, after fixation with 4% paraformaldehyde and paraffin embedding. TUNEL was performed with the ApopTag peroxidase *in situ* apoptosis detection system (Chemicon). Visualization of all nuclei was obtained by staining with Hoechst 33258 (Sigma). The number of total and TUNEL-positive nuclei for each genotype was determined in at least 300 randomly selected fields by using a Zeiss Axioplan microscope (40× magnification) equipped with a digital camera.

Statistical analysis

Except where indicated, data were analysed with the unpaired Student's *t*-test, and values with *P* < 0.05 were considered significant. Data are presented as mean ± SEM or mean ± SD.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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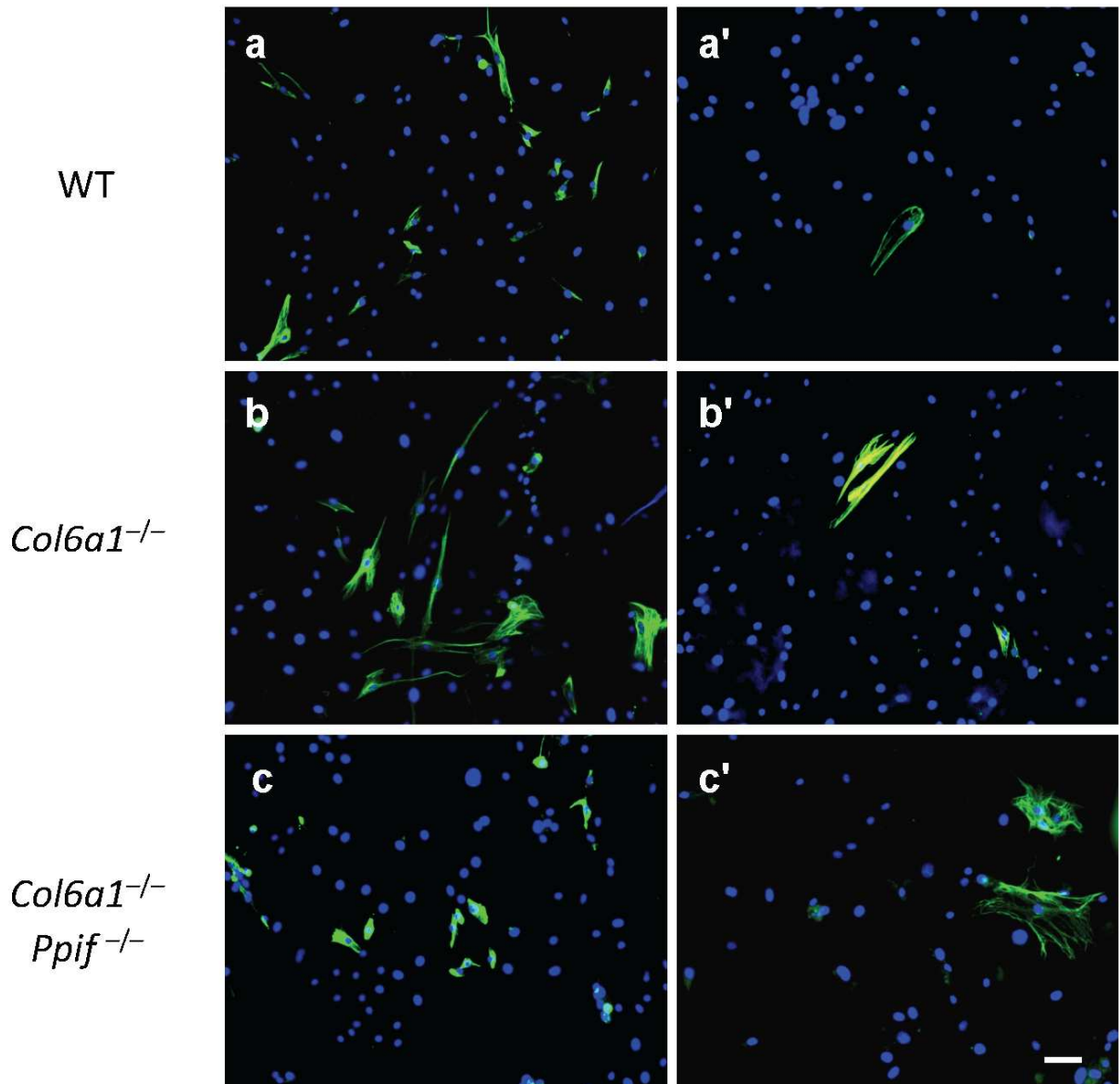
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Supplemental Material for

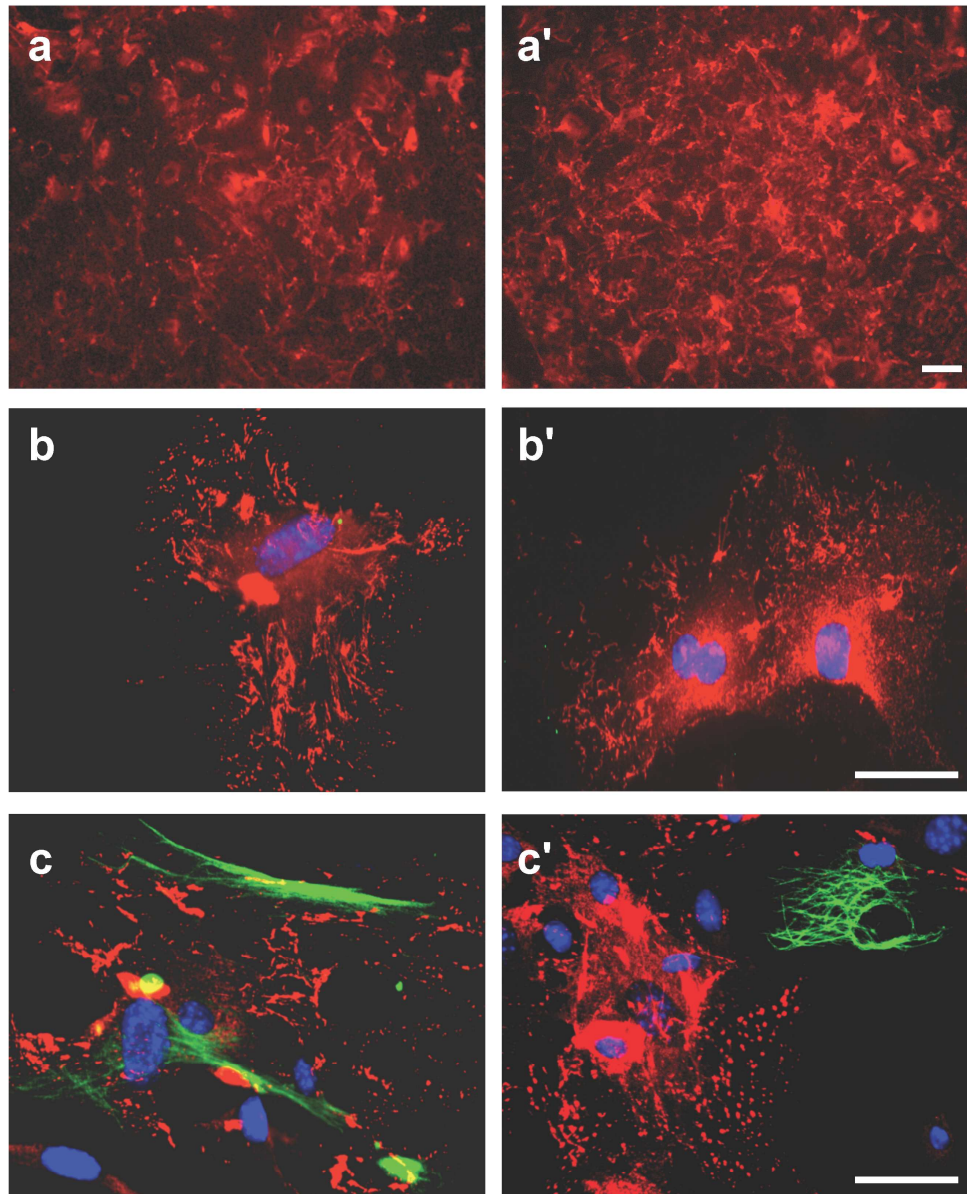
Genetic ablation of cyclophilin D rescues mitochondrial defects and prevents muscle apoptosis in collagen VI myopathic mice

Elena Palma, Tania Tiepolo, Alessia Angelin, Patrizia Sabatelli, Nadir M. Maraldi, Emy Basso, Michael A. Forte, Paolo Bernardi, Paolo Bonaldo



Supplemental Figure S1. Characterization of the primary muscle cell cultures.

Primary cultures were obtained from the diaphragms of wild-type (*a,a'*), *Col6a1*^{-/-} (*b,b'*) and *Col6a1*^{-/-}*Ppif*^{-/-} (*c,c'*) mice, as described in Material and Methods. Myoblasts were identified by immunohistochemical labeling with an antibody against desmin (green fluorescence), while nuclei were stained with Hoechst (blue fluorescence). Cultures contained a mixed population of muscle fibroblasts and myoblasts, with a comparable decrease of myoblasts after prolonged passages for all three genotypes. The panels show representative images of cultures at passage 3 (*a-c*) and at passage 9 (*a'-c'*) after initial



Supplemental Figure S2. Synthesis of collagen VI by primary muscle cell cultures obtained from wild-type mice.

Cells were grown at high density (*a,a'*) or at low density (*b,b',c,c'*) and maintained for two days in culture medium in the absence (*a-c*) or in the presence (*a'-c'*) of 0.25 mM L-ascorbic acid. Collagen VI was labeled with a polyclonal antibody (red fluorescence), while nuclei were stained with Hoechst (*b,b',c,c'*, blue fluorescence). Myoblasts were identified by immunohistochemical labeling with an antibody against desmin (*c* and *c'*,

<i>Number of culture passages after isolation</i>	<i>Percentage of desmin-positive cells</i>	<i>Percentage of TUNEL-positive nuclei</i>
3	21.78	6.31
7	11.09	1.43
9	0.18	0.37

Supplemental Table 1. Percentage of desmin-positive cells and of TUNEL-positive nuclei in *Col6a1*^{-/-} cultures at different passages.

The number of total cells, desmin-positive cells, total nuclei and TUNEL-positive nuclei was determined in at least 50 randomly selected fields, as described in Material and Methods. Cultures at lower passages show a substantial amount of myoblasts and of apoptotic nuclei, which are both markedly decreased at passages further than 7.

Supplemental Material and Methods

Immunofluorescence staining. Cells were fixed with 50% methanol/acetone for 10 min at -20° C and incubated with 10% goat serum for 30 min at room temperature. Cells were then incubated with a mouse monoclonal antibody against desmin (Sigma) and/or a rabbit polyclonal antibody against Collagen VI (Fitzgerald Industries), for 1 hour at room temperature. Reaction with secondary antibodies was carried out for 1 hour at room temperature using Alexa Fluor 488-conjugated goat anti-mouse IgG (Molecular Probes) and/or Cy3-conjugated goat-anti rabbit IgG (Jackson ImmunoResearch). Nuclei were counterstained with Hoechst 33258 (Sigma). Samples were observed with a Nikon Eclipse 80i epifluorescence microscope (10x and 40x magnifications) equipped with a digital camera.

II.

The aim of this work was to establish whether the remarkable therapeutic effects of CsA are also observed with the Cs derivative Debio 025 which maintains the ability to bind to, and inhibit, CyPs but does not inhibit calcineurin.

The results are reported in the publication: Tiepolo T, Angelin A, Palma E, Sabatelli P, Merlini L, Nicolosi L, Finetti F, Braghetta P, Vuagniaux G, Dumont JM, Baldari CT, Bonaldo P, Bernardi P (2009) The cyclophilin inhibitor Debio 025 normalizes mitochondrial function, muscle apoptosis and ultrastructural defects in Col6a1^{-/-} myopathic mice, *Br J Pharmacol* 157:1045-52, which is enclosed at pp. 52-59.

RESEARCH PAPER

The cyclophilin inhibitor Debio 025 normalizes mitochondrial function, muscle apoptosis and ultrastructural defects in *Col6a1*^{-/-} myopathic mice

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Background and purpose: We have investigated the therapeutic effects of the selective cyclophilin inhibitor D-MeAla³-EtVal⁴-cyclosporin (Debio 025) in myopathic *Col6a1*^{-/-} mice, a model of muscular dystrophies due to defects of collagen VI.

Experimental approach: We studied calcineurin activity based on NFAT translocation; T cell activation based on expression of CD69 and CD25; propensity to open the permeability transition pore in mitochondria and skeletal muscle fibres based on the ability to retain Ca²⁺ and on membrane potential, respectively; muscle ultrastructure by electronmicroscopy; and apoptotic rates by terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling assays in *Col6a1*^{-/-} mice before after treatment with Debio 025.

Key results: Debio 025 did not inhibit calcineurin activity, yet it desensitizes the mitochondrial permeability transition pore *in vivo*. Treatment with Debio 025 prevented the mitochondrial dysfunction and normalized the apoptotic rates and ultrastructural lesions of myopathic *Col6a1*^{-/-} mice.

Conclusions and implications: Desensitization of the mitochondrial permeability transition pore can be achieved by selective inhibition of matrix cyclophilin D without inhibition of calcineurin, resulting in an effective therapy of *Col6a1*^{-/-} myopathic mice. These findings provide an important proof of principle that collagen VI muscular dystrophies can be treated with Debio 025. They represent an essential step towards an effective therapy for Ullrich Congenital Muscular Dystrophy and Bethlem Myopathy, because Debio 025 does not expose patients to the potentially harmful effects of immunosuppression.

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Keywords: Mitochondria; permeability transition; cell death; cyclosporins; cyclophilins; collagen VI; muscular dystrophies

Abbreviations: CRC, calcium retention capacity; CsA, cyclosporin A; CyP, cyclophilin; Debio 025, D-MeAla³-EtVal⁴-cyclosporin; FDB, *flexor digitorum brevis*; GFP, green fluorescent protein; NFAT, nuclear factor of activated T cells; PTP, permeability transition pore; TMRM, tetramethylrhodamine methyl ester; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling; UCMD, Ullrich Congenital Muscular Dystrophy

Introduction

Mitochondria have a crucial role in pathophysiology, and the mitochondrial permeability transition pore (PTP) stands out

as an effector mechanism of cell death. Cyclosporin A (CsA) favours PTP closure after high affinity binding to cyclophilin (CyP) D, a matrix peptidyl-prolyl *cis-trans* isomerase, an effect that does not involve calcineurin (Rasola and Bernardi, 2007). In chronic diseases where the PTP plays a role, such as muscular dystrophies due to collagen VI deficiency (Irwin *et al.*, 2003; Angelin *et al.*, 2007; Merlini *et al.*, 2008) and possibly other forms of muscular dystrophy (Millay *et al.*, 2008; Reutenauer *et al.*, 2008), CsA derivatives that bind CyPs and desensitize the PTP but do not inhibit calcineurin (Nicolli *et al.*, 1996; Waldmeier *et al.*, 2002; Hansson *et al.*, 2004;

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Paeshuyse *et al.*, 2006) should therefore maintain the beneficial effects of CsA without exposing patients to the long-term complications that may arise from immunosuppression. The Cyp inhibitor D-MeAla³-EtVal⁴-cyclosporin (Debio 025) was developed from CsA by substituting Sar in position 3 and MeLeu in position 4 with D-MeAla and EtVal respectively (Hansson *et al.*, 2004). Unlike CsA, Debio 025 does not display affinity for calcineurin. *In vitro*, Debio 025 is 7000 times less effective than CsA at inhibiting interleukin-2 production by Jurkat cells and at least 15 times less potent than CsA in mixed lymphocyte reaction tests (Ptak *et al.*, 2008). In addition, Debio 025 inhibits the PTP in brain mitochondria (Hansson *et al.*, 2004), a finding that has encouraged the study of its effects in PTP-dependent disease paradigms.

In this study, we have investigated the effects of Debio 025 in myopathic *Col6a1*^{-/-} mice, a model of muscular dystrophy caused by defects of collagen VI. Our findings show that it lacks immunosuppressive activity but has therapeutic efficacy against the myopathy of this mouse disease model. Hence, they provide an important proof of principle that the *in vivo* effect of CsA is not due to inhibition of calcineurin (Irwin *et al.*, 2003), and may pave the way to the long-term treatment of Ullrich Congenital Muscular Dystrophy (UCMD) and Bethlem Myopathy.

Methods

Expression of nuclear factor of activated T cells-green fluorescent protein (NFAT-GFP)

Jurkat T cells were transfected with the plasmid enhanced green fluorescent protein (pEGFP)/NFAT-1D (Boncristiano *et al.*, 2003) using a modification of the diethylamino ethyl (DEAE)/dextran procedure described previously (Plyte *et al.*, 2001). One million Jurkat T cells were then treated for 15 min at 37°C with 0.8 μM CsA, Debio 025 or vehicle, followed by 500 ng·mL⁻¹ of the ionophore A23187. After 20 min of incubation at 37°C, the localization of NFAT-GFP was studied by laser scanning confocal microscopy (Plyte *et al.*, 2001).

Preparation of mouse T lymphocytes by negative selection

Single cell suspensions were prepared from the spleen of the 129-strain of mice using Cell Stainer-BD Falcon. Monocytes were removed by adherence. Unstimulated splenic T lymphocytes were then negatively selected by immunomagnetic sorting using anti-CD22 antibody conjugated magnetic beads (Dynabeads Mouse pan-B). Purity of T cells (typically above 90%) was assessed by flow cytometry after labelling with fluorochrome conjugated anti-CD3 and anti-CD22 mAb. Two hundred thousand T cells in 0.1 mL of RPMI-7.5% bovine calf serum were treated for 15 min at 37°C with 0.8 μM CsA or Debio 025 or with an equal volume of vehicle in six-well plates. The plates were transferred on ice, treated with saturating amounts of hamster anti-CD3 mAb (2C11) or an equivalent volume of medium (unstimulated samples) and incubated for 30 min at 4°C. Cells were washed and resuspended in 0.2 mL of cold RPMI-7.5% bovine calf serum, and 0.8 μM CsA or Debio 025, or vehicle added back to the appropriate samples, which were transferred to 96-well plates

previously coated with an antihamster secondary antibody (50 μg·mL⁻¹) and incubated at 37°C for 30 h in a CO₂ incubator. Cells were resuspended, spun, resuspended in 0.2 mL phosphate-buffered saline-1% bovine calf serum and subjected to flow cytometric analysis after being labelled with a combination of fluorochrome conjugated anti-CD3/anti-CD69 mAbs or anti-CD3/anti-CD25 mAbs.

Preparation of mitochondria and calcium retention capacity (CRC) test

Mitochondria were prepared from liver and muscle homogenates by differential centrifugation exactly as described previously (Costantini *et al.*, 1995; Fontaine *et al.*, 1998a). The CRC of mitochondrial preparations was assessed fluorimetrically in the presence of the Ca²⁺ indicator Calcium Green-5N (1 μM; excitation, 505 nm; emission, 535 nm) using a PerkinElmer LS50B spectrofluorimeter equipped with magnetic stirring and thermostatic control (Fontaine *et al.*, 1998b). The incubation conditions are detailed in the legend to Figure 3.

Isolation of skeletal myofibres and measurements of tetramethylrhodamine methyl ester (TMRM) accumulation

Fibres from *flexor digitorum brevis* (FDB) muscles were isolated exactly as described previously (Irwin *et al.*, 2002). Fibres were then plated onto glass coverslips coated with laminin (3 μg·cm⁻²) and cultured in Dulbecco's modified Eagle medium containing 10% fetal bovine serum. During the experiment, FDB fibres were placed in 1 mL Tyrode's buffer and loaded them with 20 nM of TMRM as described previously (Irwin *et al.*, 2003). The images of mitochondrial TMRM fluorescence were acquired with a Zeiss Axiovert 100 TV inverted microscope exactly as described previously (Merlini *et al.*, 2008).

In vivo treatments

Col6a1^{-/-} mice (Bonaldo *et al.*, 1998) had free access to a standard diet and were kept under controlled conditions of temperature and humidity on a 12-h light/12-h dark cycle. For the experiments presented in Figures 3–6, mice received two daily doses of Debio 025 5 mg·kg⁻¹, i.p. or an identical volume of vehicle (i.p.) for 5 days. For the experiments shown in Figure 7, the final stated doses of Debio 025 were administered through gavage in two daily doses of identical volumes. All *in vivo* experiments were approved by the competent Authority of the University of Padova and authorized by the Italian Ministry of Health.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay

We prepared 7 μm-thick sections of muscle diaphragm after 4% paraformaldehyde fixation and paraffin embedding. TUNEL was done using the ApopTag *in situ* apoptosis detection kit. Samples were stained with peroxidase-diaminobenzidine to detect TUNEL-positive nuclei and counterstained with Hoechst 33258 to identify all nuclei, as described previously (Irwin *et al.*, 2003). The total number of

nuclei and number of TUNEL-positive nuclei were determined in randomly selected fibres using a Zeiss Axioplan microscope equipped with a Leica DC 500 camera.

Electron microscopy

Diaphragm muscles from *Col6a1*^{-/-} mice were isolated and gently stretched on a dental wax support in order to prevent contraction during fixation. Samples were fixed with 2.5% glutaraldehyde in phosphate buffer 0.1 M, pH 7.4 for 3 h at 4°C, washed overnight in phosphate buffer 0.15 M, post-fixed in 1% osmium tetroxide in veronal buffer, dehydrated and embedded in Epon 812 epoxidic resin. Transverse ultrathin sections were stained with uranyl acetate and lead citrate and observed in a Philips EM400 transmission electron microscope operating at 100 kV. For statistical analysis, we studied 300 muscle fibres from three different blocks of tissue for each sample.

Data analysis and statistical procedures

Data were analysed either with the Mann-Whitney test or with Student's unpaired *t*-test as indicated in the text and legends, and only values with *P* < 0.05 were considered significant. Data are presented as mean ± SEM or mean ± SD.

Materials

Cell Stainer-BD Falcon was obtained from BD Biosciences Europe (Belgium); Dynabeads Mouse pan-B – Dynal Biotech, Invitrogen (San Giuliano Milanese, Italy); fluorochrome conjugated anti-CD3 and anti-CD22 mAb, Becton-Dickinson (Milan, Italy); Calcium Green-SN and TMRM, Molecular Probes; ApopTag *in situ* apoptosis detection kit, Intergen; Hoechst 33258, Sigma.

Results

Effects of CsA and Debio 025 on NFAT translocation and T cell activation

We investigated whether Debio 025 is able to prevent activation of calcineurin through the reporter plasmid pEGFP/NFAT-1D encoding a fusion GFP-NFAT protein that undergoes calcineurin-dependent dephosphorylation and translocation to the nucleus (Plyte *et al.*, 2001). Nuclear NFAT localization increased from 10% to about 50% after treatment with the divalent cation ionophore A23187 in a process that was blocked by CsA but not by Debio 025 (Figure 1A–C). Only CsA inhibited expression of the T lymphocyte activation markers CD69 and CD25 after stimulation with the T cell receptor agonist antibody 2C11 (Figure 2A and B respectively).

Effects of Debio 025 on the CRC of isolated mitochondria after *in vivo* administration

We next assessed whether a mitochondrial effect of Debio 025 can be demonstrated after *in vivo* drug administration to mice. Liver and hind leg muscle mitochondria were isolated from individual *Col6a1*^{-/-} mice treated for 5 days with vehicle

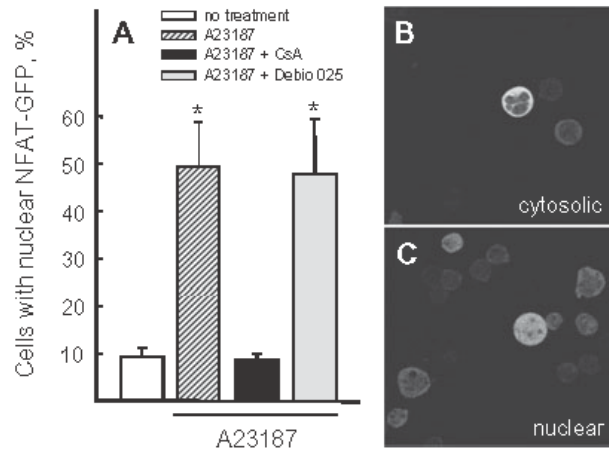


Figure 1 Effects of A23187, cyclosporin A (CsA) and Debio 025 on nuclear factor of activated T cells-green fluorescent protein (NFAT-GFP) distribution in Jurkat T cells. (A) Jurkat T cells were scored for cytoplasmic versus nuclear localization of NFAT-GFP before and after treatment with 500 ng·mL⁻¹ A23187 in the presence of vehicle, 0.8 μM CsA or 0.8 μM Debio 025. (B) and (C) Representative examples of cells with cytosolic and nuclear NFAT-GFP respectively. **P* < 0.05 versus untreated, Student's *t*-test.

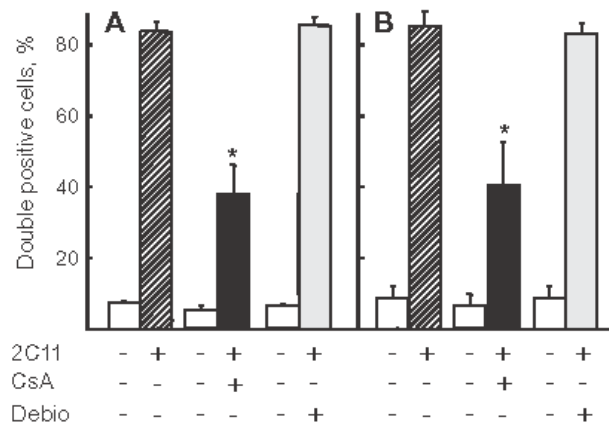


Figure 2 Effects of cyclosporin A (CsA) and Debio 025 on activation of mouse T lymphocytes. Mouse spleen CD3⁺ lymphocytes were prepared by negative selection as described in Methods and scored for expression of the activation markers CD69 (A) and CD25 (B), in the presence of vehicle, 0.8 μM CsA or Debio 025. Where indicated, cells were treated with the stimulatory antibody 2C11. **P* < 0.05 versus vehicle, Student's *t*-test.

or with Debio 025. Mitochondria were then incubated in the presence of substrates and supplemented with Calcium Green-SN, which monitors extramitochondrial Ca²⁺. A train of Ca²⁺ pulses was added, each pulse being taken up until a threshold was reached that caused opening of the PTP and precipitous release of the previously accumulated Ca²⁺ (Figure 3). The threshold Ca²⁺ needed for PTP opening was lower in liver than in muscle mitochondria (compare traces 1 in Figure 3A and A', respectively); and it was significantly increased by *in vivo* treatment with Debio 025 in both organs (traces 1 in Figure 3B and B'). The effect was particularly striking for muscle mitochondria (Figure 3B'), the CRC

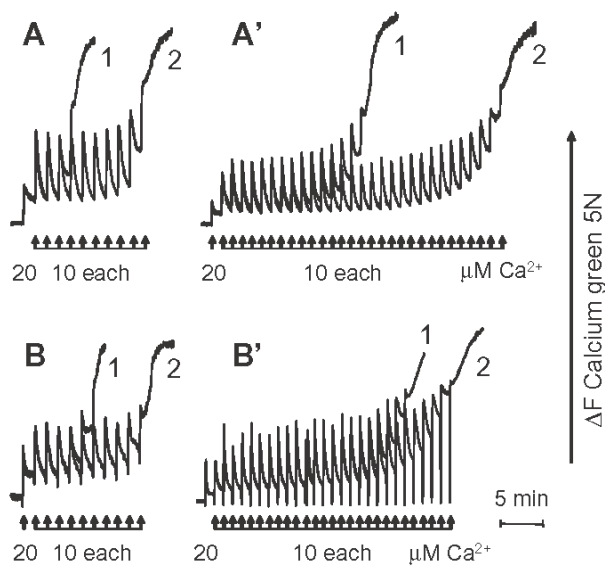


Figure 3 Effects of cyclosporin A and Debio 025 on the *in vitro* calcium retention capacity (CRC) of isolated mouse liver mitochondria. Five hours after the last injection, liver (A,B) and skeletal muscle (A',B') mitochondria were isolated from mice treated for 5 days with vehicle (A,A') or with 10 mg·kg⁻¹·day⁻¹ Debio 025 (B,B'). The incubation medium contained 0.2 M sucrose, 10 mM Tris-MOPS, 5 mM glutamate-Tris, 2.5 mM malate-Tris, 1 mM (A and B) or 10 mM (A' and B') Pi-Tris, 10 μM EGTA-Tris and 1 μM Calcium green-5N in the absence (traces 1) or presence (traces 2) of 0.8 μM Debio 025. Final volume was 2 mL, pH 7.4, 25°C. All the experiments were started with the addition of 0.5 mg·mL⁻¹ mitochondria (not shown) followed 1 min later by the indicated pulses of Ca²⁺. Traces are representative of four replicates for each condition. (C) Shows the CRC/CRCmax of liver and skeletal muscle mitochondria for each individual animal (dot) treated with vehicle or Debio 025. Average values for Debio 025-treated are significantly different from those of vehicle-treated animals ($P < 0.05$, Student's *t*-test).

getting close to the maximum level that could be attained by adding Debio 025 directly to the cuvette (trace 2 in all panels). Statistical analysis revealed a highly significant desensitization of the PTP in both liver and muscle mitochondria (Figure 3C). It should be noted that the CRC measured in mitochondria treated with Debio 025 *in vivo* represents a

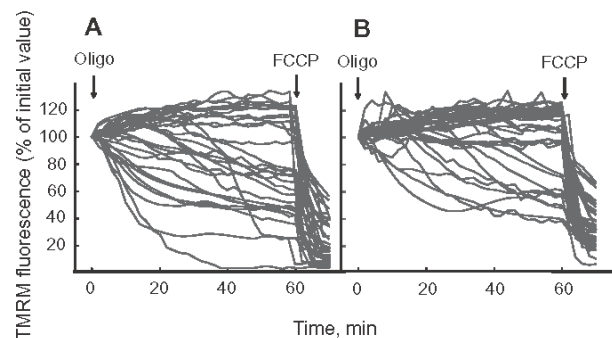


Figure 4 Effects of oligomycin and carbonylcyanide-*p*-trifluoromethoxyphenyl hydrazone (FCCP) on mitochondrial tetramethylrhodamine methyl ester (TMRM) fluorescence in *flexor digitorum brevis* (FDB) fibres isolated from *Col6a1*^{-/-} mice treated with vehicle or with Debio 025. Mice were treated for 5 days with vehicle (A) or with 10 mg·kg⁻¹·day⁻¹ Debio 025 (B). FDB fibres were isolated and mitochondrial fluorescence images acquired as described in Methods. Where indicated, 5 μM oligomycin (Oligo) and 4 μM FCCP were added. Each trace shows the fluorescence of a single fibre, and data from all treated animals were pooled.

lower limit, because some washout effect was expected to occur during the preparation of mitochondria.

Effects of *in vivo* administration of Debio 025 on

oligomycin-dependent mitochondrial depolarization in FDB fibres
Next, we tested whether this pharmacological inhibition of the PTP can be demonstrated in FDB fibres isolated from Debio 025-treated *Col6a1*^{-/-} mice. In order to explore the status of the PTP, we exploited our previous observation that most of these fibres display an anomalous depolarizing response to the F1FO ATPase inhibitor oligomycin, which can be corrected by CsA (Irwin *et al.*, 2003). Addition of oligomycin to vehicle-treated animals caused decreased fluorescence of mitochondrial TMRM (a measure of mitochondrial depolarization) in a fraction of the fibres; if a threshold is arbitrarily set at 90% of the initial fluorescence value (Merlini *et al.*, 2008), 18 out of 31 fibres (58%) and 10 out of 40 fibres (25%) depolarized in vehicle- and Debio 025-treated animals respectively (Figure 4, compare A and B).

Effects of Debio 025 on mitochondrial ultrastructural defects and diaphragm apoptosis *in vivo*

Swelling, a typical feature of mitochondrial PTP opening, was easily detected in *Col6a1*^{-/-} (Figure 5A) but not in wild-type diaphragm fibres (results not shown), and mitochondrial ultrastructure was normalized by treatment with Debio 025 (Figure 5B). Debio 025 caused a reduction in the number of abnormal mitochondria in all individuals (Figure 5C) with an average decrease of affected fibres from 13 to 2.5 % (Figure 5D). We also assessed whether treatment with Debio 025 was able to normalize the increased apoptotic rates observed in *Col6a1*^{-/-} mice (Irwin *et al.*, 2003). Vehicle-treated animals had an average of 55.4 ± 7.3 apoptotic nuclei per mm², which is well within the range previously reported for these mice (Irwin *et al.*, 2003). After treatment with Debio

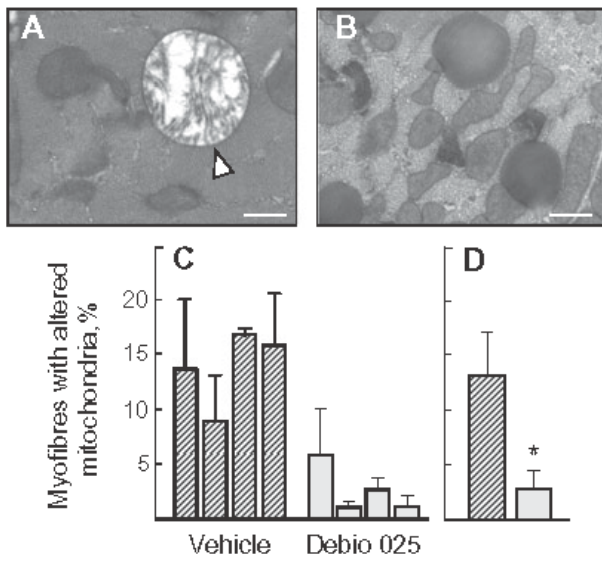


Figure 5 Ultrastructure of mitochondria in diaphragms from *Col6a1*^{-/-} mice treated with vehicle or with Debio 025. Cross sections of diaphragms from *Col6a1*^{-/-} mice treated for 5 days with vehicle (A) or Debio 025 (B) were analysed. Swollen mitochondria were easily detected in *Col6a1*^{-/-} muscle fibres from mice treated with vehicle (arrowhead in A). Bar, 400 nm. In (C) and (D) the ordinate shows the percent of myofibers with altered mitochondria observed in diaphragm sections from *Col6a1*^{-/-} mice treated with vehicle or with Debio 025. (C) Each column refers to one individual; (D) average. **P* < 0.05 versus vehicle, Mann-Whitney test.

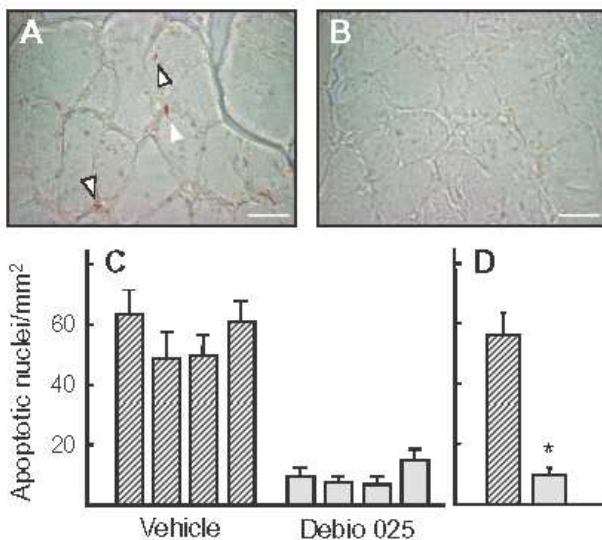


Figure 6 Incidence of apoptosis in diaphragms from *Col6a1*^{-/-} mice treated with vehicle or with Debio 025. Cross sections of diaphragms from *Col6a1*^{-/-} mice treated for 5 days with vehicle (A) or Debio 025 (B) were analysed. Apoptotic nuclei were easily detected in *Col6a1*^{-/-} muscle fibres from mice treated with vehicle (arrowheads in A). Bar, 50 μ m. In (C) and (D) the ordinate scale represents the number of apoptotic nuclei per mm² observed in diaphragm sections from *Col6a1*^{-/-} mice treated with vehicle or with Debio 025. (C) Each column refers to one individual; (D) average. **P* < 0.05 versus vehicle, Student's *t*-test.

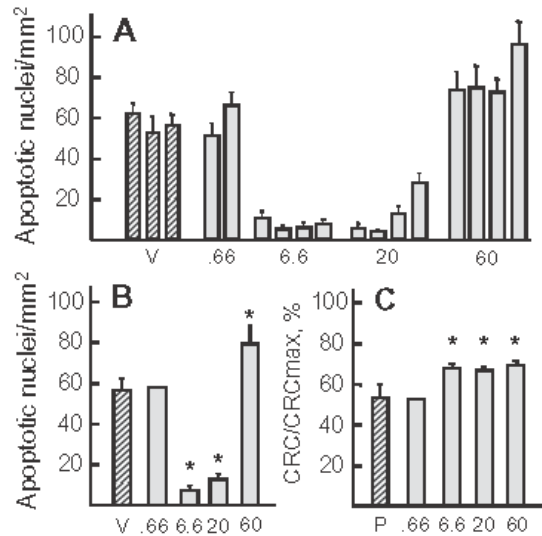


Figure 7 Dose-dependence of the effects of Debio 025 on diaphragm apoptosis and skeletal muscle calcium retention capacity (CRC) in *Col6a1*^{-/-} mice. (A) and (B) The ordinate scale represents the number of apoptotic nuclei per mm² observed in diaphragm cross sections from *Col6a1*^{-/-} mice treated with vehicle (V) or with Debio 025 (dose is in mg·kg⁻¹·day⁻¹). Between 20 and 30 sections per mouse were analysed. (A) Each column refers to one individual; (B) average. (C) Represents the average CRC/CRCmax of skeletal muscle mitochondria prepared from the same individuals treated with vehicle (V) or Debio 025. **P* < 0.05 versus vehicle, Student's *t*-test.

025, the incidence dropped to 9.5 ± 2.6 nuclei per mm² (Figure 6), which is the same value reported after treatment with CsA (Irwin *et al.*, 2003). We finally studied the dose dependence of the effects of Debio 025 on diaphragm apoptosis after gavage. It can be seen that $0.66 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ was ineffective, while doses of 6.6 and $20 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ day effectively reduced the number of apoptotic nuclei, consistent with the CRC increase measured in muscle mitochondria (Figure 7). Doses of $60 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ were no longer effective at preventing apoptosis, but rather slightly increased the frequency of apoptotic nuclei (Figure 7 A,B); yet the effect of Debio 025 $6.6 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ on the CRC was indistinguishable from that of $60 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ (Figure 7C). The overall increase of CRC was somewhat lower than that observed after i.p. administration of Debio 025 (compare with Figure 3C).

Discussion and conclusions

CyPA is the prototype of the CyP family of peptidyl prolyl *cis-trans* isomerases (Fischer *et al.*, 1989), which in humans includes 17 unique proteins (Wang and Heitman, 2005). After binding to its inhibitory ligand CsA (Borel *et al.*, 1977), the CsA/CyPA complex binds to and inhibits the cytosolic phosphatase calcineurin (Liu *et al.*, 1991) resulting in immunosuppression (Clipstone and Crabtree, 1992; Walsh *et al.*, 1992). Early work with active site mutants of human CyPA had already clearly separated the peptidyl prolyl *cis-trans* isomerase activity of the protein from CsA binding and calcineurin inhibition (Zydowsky *et al.*, 1992), suggesting that

CyPs have cellular functions that are independent of immunosuppression. This prediction has been fulfilled, and the emerging field of CyP biochemistry and pathophysiology is uncovering the great importance of these proteins for a variety of processes relevant to human disease (Wang and Heitman, 2005). These include inflammation and vascular dysfunction (Jin *et al.*, 2004; Kim *et al.*, 2004; 2005; Arora *et al.*, 2005; Damsker *et al.*, 2007), wound healing (Kong *et al.*, 2007), innate immunity to HIV (Sokolskaja and Luban, 2006), hepatitis C infection (Flisiak *et al.*, 2007), host-parasite interactions (Bell *et al.*, 2006), tumour biology (Yao *et al.*, 2005) and mitochondrial PTP-dependent dysfunction mediated by matrix CyPD (Connern and Halestrap, 1992; Nicolli *et al.*, 1996; Woodfield *et al.*, 1997; Waldmeier *et al.*, 2003). These studies underline the importance of developing CyP ligands like Debio 025 that do not result in formation of calcineurin-inhibitory complexes and thus offer great promise for the therapy of CyP-dependent diseases such as HCV and HIV (Flisiak *et al.*, 2008; Paeshuyse *et al.*, 2006; Inoue *et al.*, 2007; Ptak *et al.*, 2008) and collagen VI muscular dystrophies (present manuscript).

We have shown (i) that Debio 025 does not inhibit calcineurin-dependent NFAT translocation to the nucleus in Jurkat T cells, nor does it prevent activation of resting mouse T lymphocytes; this is conclusive evidence that calcineurin is not involved in the protective effects displayed by CsA in the *Col6a1*^{-/-} mouse (Irwin *et al.*, 2003), an issue of importance because calcineurin affects mitochondrial fission through dephosphorylation of Drp-1 (Cereghetti *et al.*, 2008); (ii) that this drug is an effective inhibitor of the PTP *in vivo*, as demonstrated by its desensitizing effects in mitochondria and FDB myofibres isolated from Debio 025-treated mice; and (iii) that treatment of *Col6a1*^{-/-} mice with Debio 025 has a therapeutic efficacy matching that previously described with CsA (Irwin *et al.*, 2003). Remarkably, and at variance from the decreased contractile performance of human and rabbit heart muscle preparations caused by CsA (Janssen *et al.*, 2000), Debio 025 was cardioprotective in a mouse model of myocardial infarction (Gomez *et al.*, 2007) suggesting that the toxic effects of CsA are rather due to inhibition of calcineurin.

It should also be noted that Debio 025 was effective after both i.p. and oral administration, and that a *per os* dose of 6.6 mg·kg⁻¹·day⁻¹ was extremely effective at inhibiting diaphragm apoptosis, with no further decrease at 20 mg·kg⁻¹·day⁻¹. The antiapoptotic effect was not observed at 60 mg·kg⁻¹·day⁻¹, a dose that slightly increased the incidence of apoptosis. This event appears to be independent of PTP desensitization because the CRC of skeletal muscle mitochondria was the same at 6.6, 20 and 60 mg·kg⁻¹·day⁻¹ of Debio 025. This point should be considered in the future use of Debio 025 in a clinical setting for muscular dystrophy, and deserves further study. It should be mentioned, however, that Debio 025 1000 mg daily is in general well tolerated and proved to be safe in HCV-infected patients, the most prominent side effect being transient hyperbilirubinemia caused by inhibition of biliary canalicular transporters (Flisiak *et al.*, 2009).

Debio 025 inhibits all CyPs including CyPA and/or B, which appears to be responsible for the effects of the drug on HCV and HIV replication respectively (Chatterji *et al.*, 2005;

Bobardt *et al.*, 2008; Kaul *et al.*, 2008). The conclusion that Debio 025 prevents muscle cell apoptosis in the *Col6a1*^{-/-} mouse through CyPD inhibition and PTP desensitization is considerably strengthened by our recent finding that genetic ablation of CyPD cures the myopathy of *Col6a1*^{-/-} mice (Palma *et al.*, 2009).

Taken together, our findings have major implications for UCMD patients, whose treatment with CsA has proven beneficial (Merlini *et al.*, 2008). UCMD is a chronic muscle-wasting disease involving the diaphragm, and respiratory failure is a common complication which is worsened by pulmonary infections (Merlini and Bernardi, 2008). Long-term treatment with CsA must therefore be carefully weighed against the risks of immunosuppression, which may favour life-threatening infections. Debio 025 has already proved effective at restoring mitochondrial function and at decreasing apoptosis in myoblasts from patients affected by UCMD and Bethlem myopathy (Angelin *et al.*, 2007). Since this drug is as effective as CsA in the *Col6a1*^{-/-} mouse animal model, we believe that the present results represent an important and necessary step towards a therapy of human collagen VI muscular dystrophies with Debio 025.

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Conflict of interest

The Authors declare no conflict of interest.

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III.

The aims of this work were to assess whether the anomalous depolarizing response to oligomycin, which we have observed in cells from *Col6a1*^{-/-} mice and patients affected by UCMD, is a distinctive feature of ColVI-related disorders, a point that has been questioned in a recent publication [79]; and whether mitochondrial respiration is impaired in these pathologies.

Material and methods

Participants

BM and UCMD were diagnosed according to the criteria of the European Neuromuscular Center [148]. All patients were examined and all underwent a muscle biopsy. The basic features of the patients analyzed in this study are summarized in Table 1. All participants provided written informed consent, and approval was obtained from the Ethics Committee of the Rizzoli Orthopedic Institute (Bologna, Italy).

PAT	PHENOTYPE	AGE	COLLAGEN VI	MUTATION
1	BM-walker	31yr	Normal in skin, cultured fibroblasts and skeletal muscle	31 aa, COL6A3, exon 17 c. 6485 G>A, het, G2077D, missense
2	BM-walker	57yr	Normal in muscle fibers, decreased in fibroblasts	COL6A1 heterozygous Tyr122-Gly143del
3	BM-walker	7yr	Normal in skin, cultured fibroblasts and skeletal muscle	no mutations in COL6A1, COL6A2, COL6A3
4	BM-walker	17yr	Normal in skin, cultured fibroblasts and skeletal muscle	16 aa, COL6A2 exon 26, c. 2180 G>A, het, G700S, missense (de novo)
5	UCMD-nonwalker	6yr	marked reduction in muscle fibers	COL6A2 compound heterozygous Gly487-Ala495delAspfsX48 and Glu591-Cys605delThrfsX148

Table 1. Features of BM and UCMD patients.

Primary muscle-derived cell cultures

We obtained muscle biopsies from healthy donor, BM and UCMD patients after obtaining the informed consent of patients and approval of the ethics committee of the Rizzoli Orthopedic Institute (Bologna, Italy). Cell cultures were established from muscle biopsies of one unaffected control and from patients 1–5. Cell cultures were carried out using enzymatic and mechanical treatment of muscle biopsies and plating in Dulbecco's modified Eagle's medium (DMEM) plus 20% fetal calf serum (FCS) and antibiotics (penicillin, streptomycin, and amphotericin B -Sigma, St. Louis, MO) and then stored in liquid nitrogen.

Immunofluorescence on cell cultures

Muscle-derived cell cultures were fixed with cold methanol, washed with PBS, and incubated with desmin antibody (Abcam). The immunoreaction was revealed with TRITC-conjugated antibody (DAKO), mounted with Pro-long anti-fade reagent (Molecular probes) and examined with Nikon epifluorescence microscope at X100 magnification.

TMRM assay

Mitochondrial membrane potential was measured as described in [142].

TUNEL assay

We measured the rate of apoptosis in primary muscle-derived cell cultures using the terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) method. Cell cultures were seeded onto Lab-Tek plastic chamber slides (Nunc, Roskilde, Denmark) and grown to confluence in DMEM supplemented with 20% FCS. Cells were fixed in 50% acetone/50% methanol and processed for TUNEL analysis by using the DeadEnd Fluorometric TUNEL System (Promega, Madison, WI). Visualization of all nuclei was performed by staining with Hoechst 33258 (Sigma). The number of total and TUNEL-positive nuclei was determined in randomly selected fields by using a Zeiss (Oberkochen, Germany) Axioplan microscope (X40 magnification) equipped with a digital camera. Data are expressed as mean \pm SD. Data were analyzed with the unpaired Student's *t* test, and values of $P < 0.01$ were considered significant.

Measurement of mitochondrial respiration

Oxygen consumption rate (OCR) was measured using *XF24 Extracellular Flux Analyzer* (Seahorse Bioscience, N. Billerica, MA- Figure 16A). This instrument measures in real time the extracellular flux changes of oxygen and protons in the media (DMEM -Sigma D5030- added with 25 mM glucose, 1 mM sodium pyruvate and 2 mM glutamine) immediately above adherent cells cultured in a microplate. In a typical experiment, cells that had been grown in flasks were trypsinized and counted, and then ~20,000 cells per well were seeded in appropriate 24-well plates, each with 28 mm² surface and containing ~900 μ l media (Figure 16B).

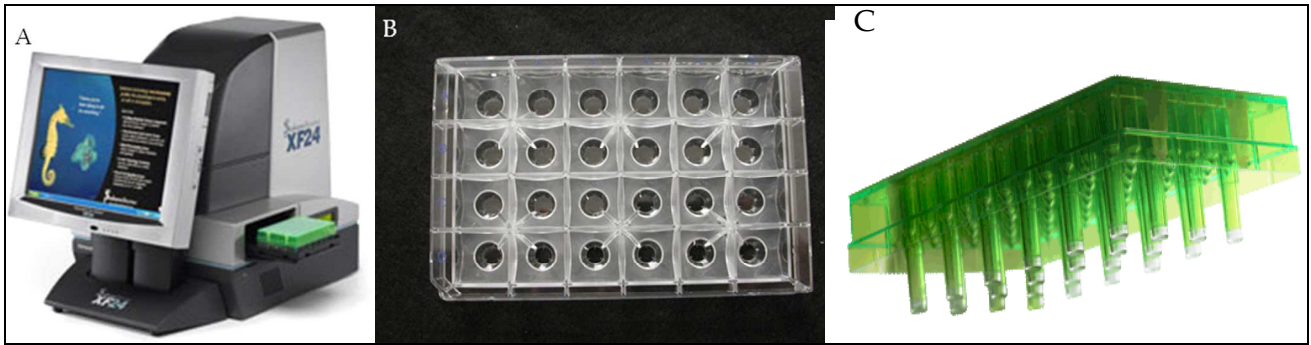


Figure 16. XF24 Extracellular flux Analyzer (A), a 24-well microplate (B) and a sensor cartridge (C).

Each XF24-microplate is coupled to a disposable sensor cartridge (Figure 16C), embedded with 24 pairs of probes for measuring oxygen concentration and pH. The probes are embedded at the tip of the 24 pistons of the cartridge and are coupled to fiber-optic waveguides. The waveguide delivers light at various excitation wavelengths (oxygen = 532 nm, pH = 470 nm) and transmits a fluorescent signal, through optical filters (oxygen = 650 nm, pH = 530 nm) to a set of highly sensitive photo detectors. Each fluorophore is uniquely designed to measure a particular analyte. For example, the oxygen concentration is measured as the quenching of fluorescence from a porphyrin-based fluorophore specific for oxygen embedded in a polymer at the tip of the disposable cartridge. Each sensor cartridge is also equipped with four reagent delivery chambers per well for injecting testing agents into wells during an assay (Figure 17). Prior to the start of the XF assay, the array of biosensors is independently calibrated using an automated routine that determines a unique sensor gain based on the sensor output measured in a calibration reagent of known pH and oxygen concentration. During the assay, after 3 minutes mixing, the sensor cartridge is lowered onto the wells to reach the measure position. Lowering the probe head, which is parked at 250 μm above the cell monolayer, reduces the volume in which cells are contained to $\sim 7 \mu\text{l}$ of media (Figure 17). The OCR is then measured during 2 minutes, after which the cartridge is lifted and the cells exposed to fresh media. The cycle is repeated three times at 5-minute intervals.

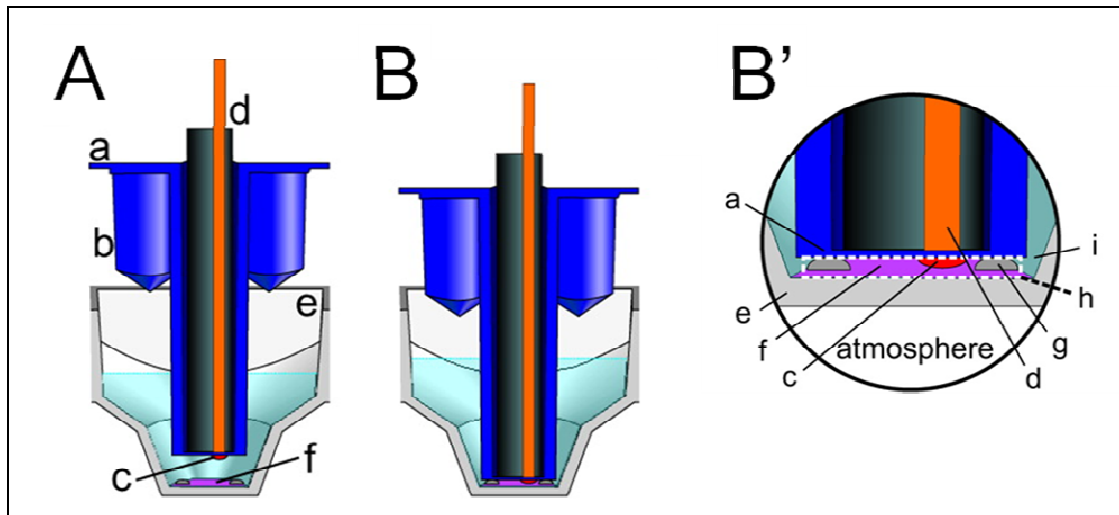


Figure 17. Seahorse XF24 V7 microplate chamber and the generalized compartment model for semiclosed chamber oxygraphs. (A and B) Well of the XF24 microplate, with probe in up (A) and in measure (B and B') positions. (a; blue) disposable cartridge consisting of the drug injection ports (b) and the O₂ probe (c; red). (d) steel rod containing fiber optics for fluorescence data collection. (e; gray) one well of the flux plate with assay medium and the monolayer of cells (f; purple). (g) Bumps in the bottom of the well acting as spacers establishing the thickness (200 μm) of the entrapped volume denoted as "chamber" (h; dashed white outline). The entrapped volume is open from the sides, but slow diffusion rates limit O₂ exchange with fluid spaces (i) around the rim of the tip of the sensor piston (a). Modified from [65].

Upon completion of an XF experiment, we assessed cell viability, and counted cells, using the calcein AM (Molecular Probes) assay directly on the 24-wells (Figure 18). The values of OCR reflect both the metabolic activities of the cells and the number of cells being measured. The number of cells present in a well is relevant since different cell populations are compared.

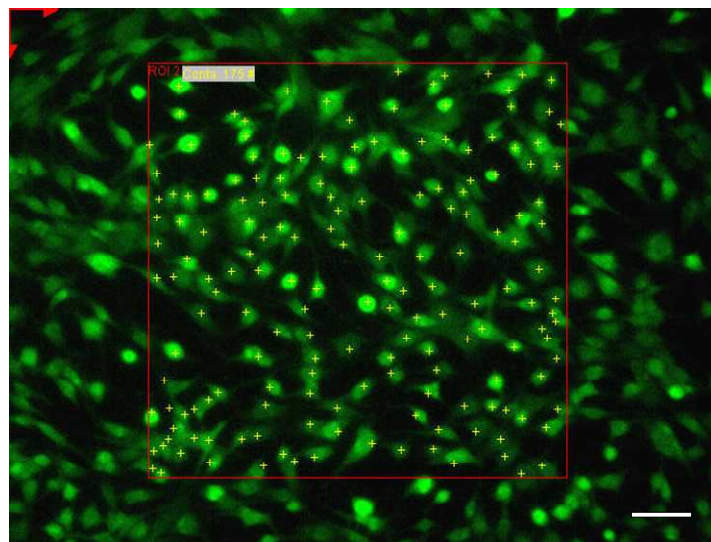


Figure 18. Calcein AM staining and count of cells after XF experiment. After the measurement of mitochondrial respiration, cells were stained with Calcein AM to evaluate the number of viable cells per well. Images of cells were acquired with an Olympus IX71/IX51 inverted microscope, equipped with a xenon light source for epifluorescence illumination, and with a 12 bit digital cooled CCD camera (Micromax, Princeton Instruments). For detection of fluorescence, 488±25 nm bandpass excitation and 525 nm longpass emission filter settings were used. Images were collected using 10X objective (Nikon). Data were acquired and analyzed using Cell R software (Olympus). The image is representative of cells loaded with Calcein AM. The red square defines a region in the well with 0,25 mm² surface. It was used to obtain the total number of viable cells per well at the end of the experiment. In yellow is shown the count of cells inside the red square. Bar, 50 μm.

Results

The five patients we studied are representative of the clinical and molecular variety of ColVI-related disorders, they have different age and type of COL6 genes mutations. Four were affected by BM and one by UCMD; two were of pediatric age and three were adults (Table 1). We established primary cell cultures from muscle biopsies and studied mitochondrial function. 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 [139]. As already discussed [5;88;126;142], cells were 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 [21]. In our previous work [5], we demonstrated that addition of the F_1F_0 ATP synthase inhibitor oligomycin to cultures established from healthy donors did not cause mitochondrial depolarization, which was instead promptly caused by the addition of the protonophore carbonylcyanide-*p*-trifluoromethoxyphenyl hydrazone (FCCP). Here we show that addition of oligomycin is instead followed by mitochondrial depolarization in cells from BM patients (Figure 19), as previously observed also in cells from UCMD patients [5].

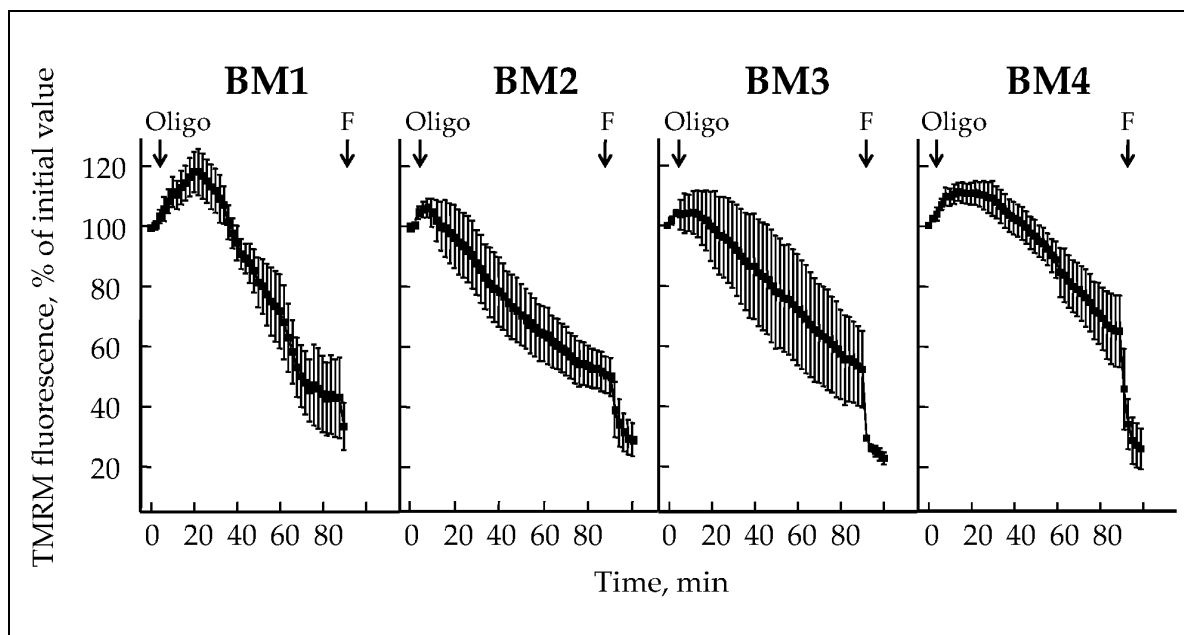


Figure 19. Mitochondrial response to oligomycin in primary muscle-derived cultures from BM patients.

Cells from four patients affected by BM were studied as described in “Material and methods”. When indicated by arrows, 6 μ M oligomycin (Oligo) and 4 μ M FCCP (F) were added. Data represent the mean of at least four independent experiments per patient \pm SEM.

Then, we investigated the mitochondrial response to rotenone (inhibitor of complex I) in one BM patient and we obtained the same results found in a UCMD patient [4]. Indeed,

just few minutes after the addition of rotenone, the membrane potential of mitochondria in BM cultures decreased very rapidly (Figure 20).

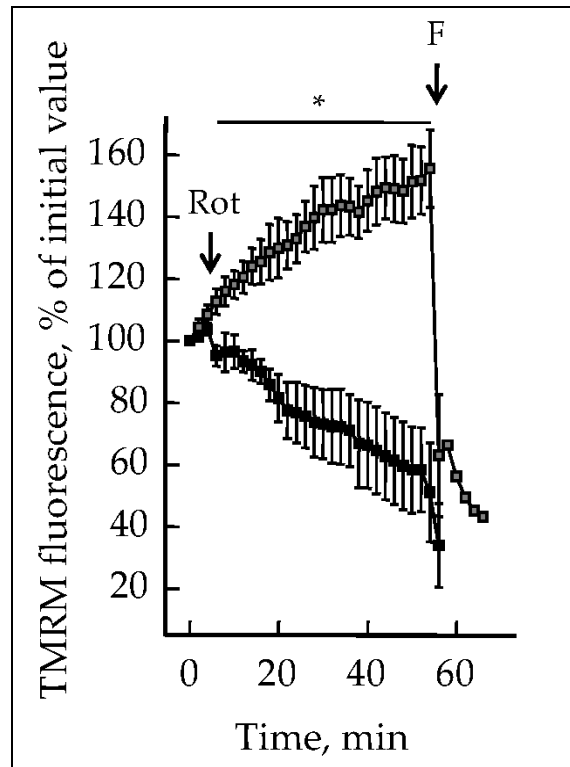


Figure 20. Changes of mitochondrial TMRM fluorescence induced by rotenone in BM cells.

Cell culture established from muscle biopsy of a BM patient (n.1 in Table 1) were loaded with TMRM and studied as described in “Material and methods”. Where indicated by arrows 4 μM rotenone (Rot; black squares only) and 4 μM FCCP (both traces) were added. The means of at least three experiments \pm SEM are shown. The upper bar indicates all the time points where $P < 0.05$ (*) for cells treated with rotenone (black squares) compared with cells without any treatment (grey squares).

This finding confirms our previous discovery that also rotenone unmasks the latent mitochondrial dysfunction causing depolarization in mitochondria from patients with ColVI myopathies. In our previous work [4], we had demonstrated that in healthy cells rotenone addition caused only a slight decrease of the mitochondrial membrane potential. Thus, our hypothesis is that in these cells depolarization was prevented by reversal of the ATP synthase and that the threshold voltage for PTP opening was not reached. The permeability of the IMM remained low and the membrane potential was maintained at the expense of ATP hydrolysis. On the other hand, in UCMD and BM patients we have mitochondrial depolarization because the PTP threshold is instead very close to the resting membrane potential, probably as a result of increased matrix Ca^{2+} levels and/or of PTP sensitization to other factors. This condition leads to PTP opening even for small depolarizations, as for example that caused by the rotenone inhibition of mitochondrial respiration (Figure 21).

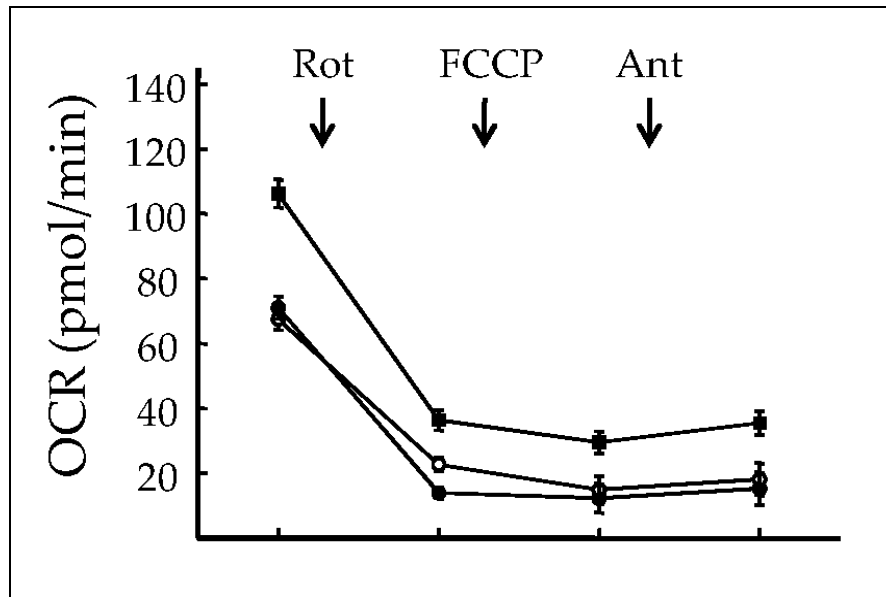


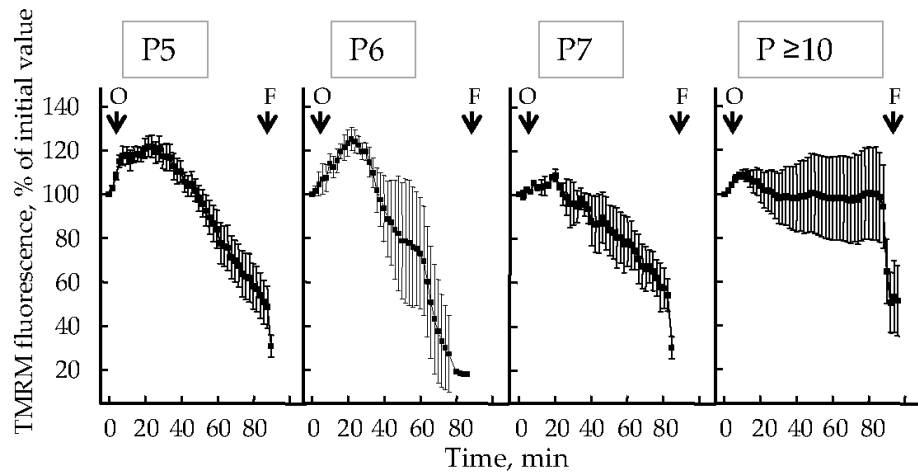
Figure 21. Oxygen consumption rate (OCR) of cells from one healthy donor, one UCMD and one BM patient.

The OCR was measured in intact cells as described in “Material and methods”. Where indicated by arrows 4 μ M rotenone (Rot), 0,3 μ M FCCP and 1,8 μ M antimycin (Ant) were added. The addition of rotenone to cells caused the inhibition of respiration in healthy donor (closed squares), as well as in UCMD patient (closed circles) and in BM patient (open circles). The data are the mean of three wells of cells \pm S.D.

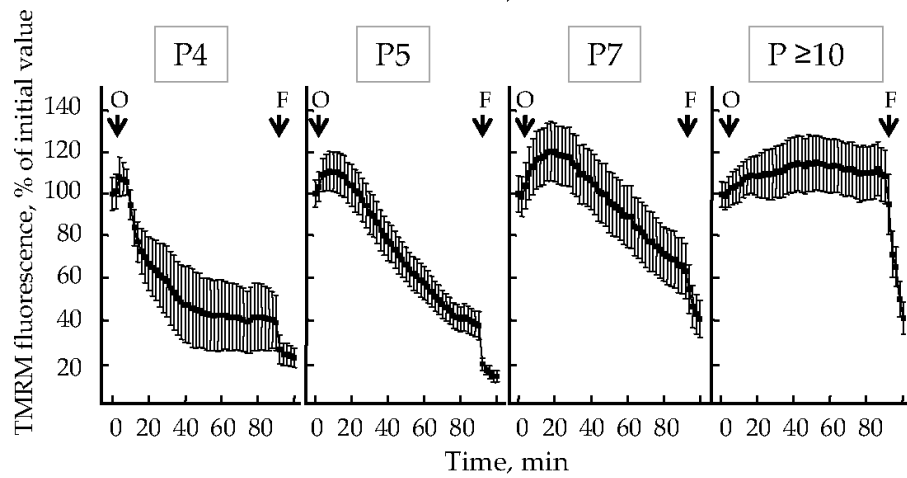
Our data on mitochondrial membrane potential confirm that cells from patients affected by ColVI muscular dystrophies have a latent mitochondrial dysfunction that can be unmasked by oligomycin (or by rotenone), a point that has been questioned in a recent publication [79]. The authors have studied the mitochondrial membrane potential in muscle-derived cultures from patients affected by different forms of muscular dystrophy. Mitochondrial depolarization was found in two UCMD patients, in keeping with our results, and also in cells derived from a patient with limb-girdle muscular dystrophy (LGMD2B). A pathogenic role for mitochondria in muscle myopathies was, moreover, suggested also by other groups [131;156].

However, in the work mentioned above [79], oligomycin-induced mitochondrial depolarization was not observed in cells derived from one BM patient, and this result is in contrast with our study reported above. In order to find a possible explanation, we have characterized the primary cells cultures we use, and their response to apoptotic stimuli according to the time in culture (number of passages). We have noticed that the response to oligomycin or rotenone observed in UCMD and BM patients was closely related to the age of the culture, and that cells displayed a different sensitivity to these stimuli according to the number of cell passages. We observed that after 7 passages in culture the mitochondria of UCMD and BM patients did not depolarize after oligomycin addition, while they did so at earlier passages (Figures 22 and 23).

BM1



BM2



UCMD

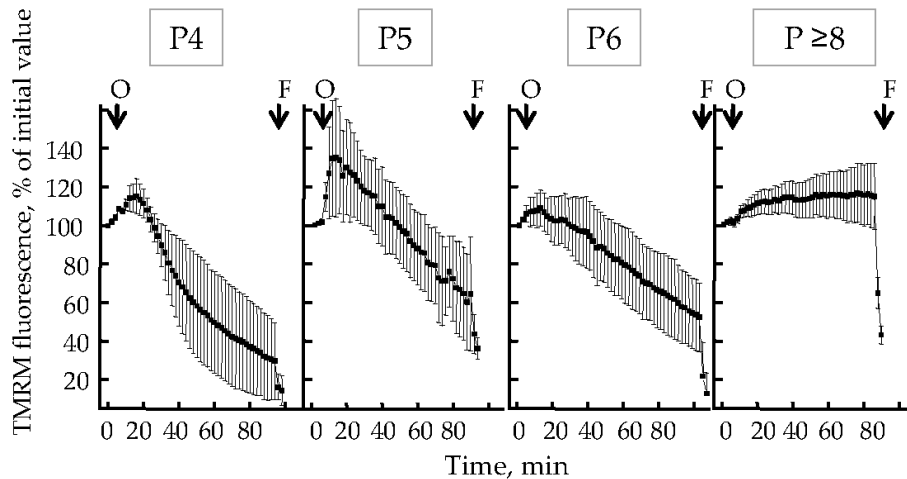


Figure 22. Changes of mitochondrial TMRM fluorescence after oligomycin addition in cell cultures from BM and UCMD patients.

Experiments with cells from BM patient n. 1 and n. 2, and UCMD patient are divided according to the number of cell passages (indicated by P). Where indicated by arrows 6 μ M oligomycin (O) and 4 μ M FCCP (F) were added. Each trace represents the mean \pm SEM of at least three experiments per passage.

We also analyzed the mitochondrial response after the addition of rotenone, and cells after 7 passages in culture became insensitive also to this stimulus (Figure 23). These findings demonstrate that the mitochondrial phenotype can dramatically change according to the

number of cell passages; and that the depolarizing effects of oligomycin and rotenone can be completely lost both in UCMD and in BM patients. It should be noted that in the study of Hicks *et al.* the cell passage number was not disclosed; we suspect that the discrepancy with our results may depend on this issue. It is reassuring, however, that the mitochondrial phenotype observed *in vivo* is invariably present in the early cultures, which are presumably the closest representation of the *in situ* situation.

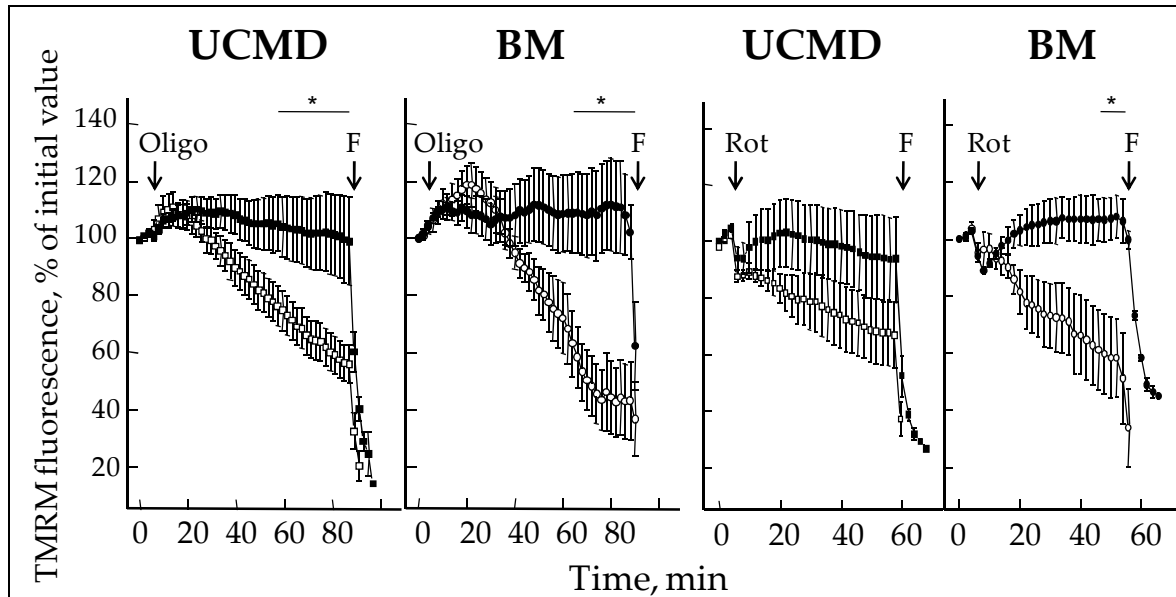


Figure 23. Effect of oligomycin and rotenone on mitochondrial membrane potential in UCMD and BM patients. Open symbols represent experiments performed in cells with number of passages ≤ 7 ; closed symbols in cells with number of passages ≥ 9 . Where indicated by arrows 6 μM oligomycin (Oligo) and 4 μM FCCP (F) were added. Traces are the mean of at least four experiments \pm SEM. The upper bars indicate all the time points where $P < 0.05$ (*).

We evaluated whether the level of apoptosis in UCMD cultures also changed according to the number of cell passages. Interestingly, we found that the percentage of apoptotic nuclei abruptly decreased after 10 passages in culture, reaching the same level of healthy donors (about 0,5%, [5]) (Figure 24).

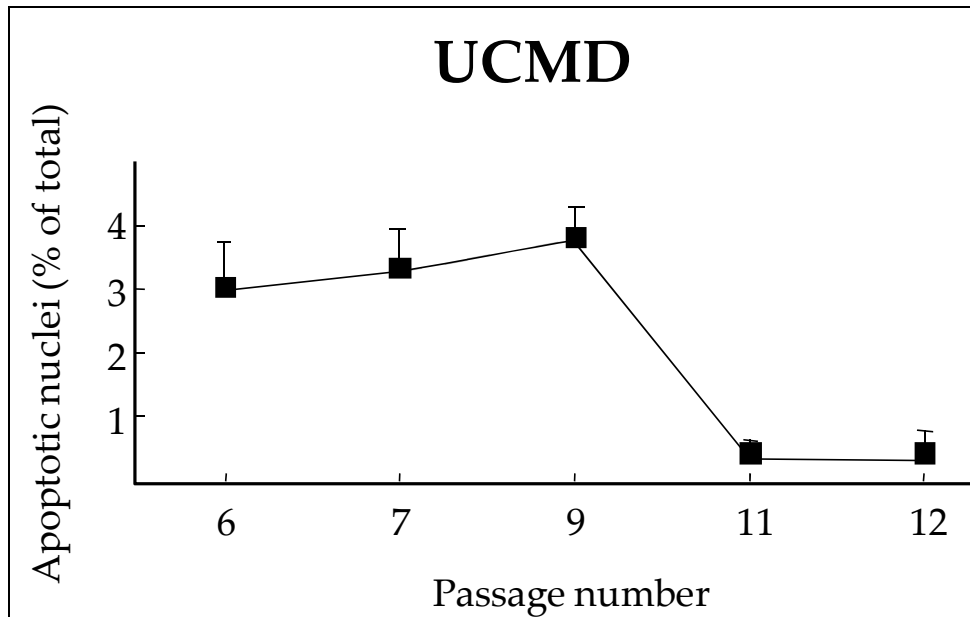


Figure 24. Incidence of apoptosis in muscle-derived cell culture from a UCMD patient.

The percentage of apoptotic nuclei were determined by TUNEL assay as written in "Material and methods". Data are the mean of at least four experiments \pm SEM and represent the level of apoptosis in cultures at the indicated number of cell passages.

These findings further confirm that muscle-derived cells from patients kept in culture for many passages lost their characteristic phenotype and became resistant, or less sensitive, to apoptotic stimuli. It should be mentioned, that our primary muscle-derived cell cultures contain different cell types, including myoblasts (identified as desmin-positive cells) and muscle fibroblasts. We have previously demonstrated that in primary muscle-derived cell cultures from diaphragm of mice the percentage of desmin-positive cells decreased very rapidly during cell passages and that cultures after about 9 passages were composed almost totally by fibroblasts (pp.47, 49). Thus, we decided to evaluate whether muscle fibroblasts depolarized after the addition of oligomycin, since dermal fibroblasts from UCMD patients did not [79]. We performed the TMRM assay on cells seeded on coverslips with a marked grid, so that at the end of the experiment we could test the cells we had studied for the presence of muscle proteins. After the evaluation of the response to oligomycin, we identified the myoblasts by immunohistochemical labeling with an antibody against desmin. As shown in Figure 25, desmin-negative cells (presumably fibroblasts) depolarized after the addition of oligomycin in cultures from UCMD and BM patients.

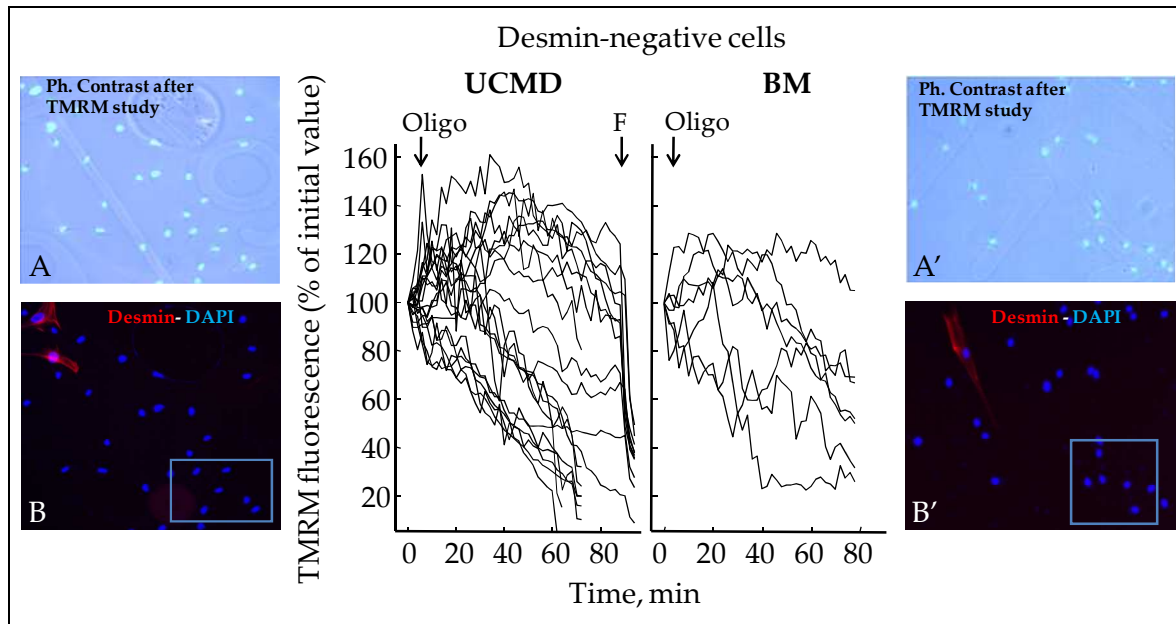


Figure 25. Analysis of mitochondrial response to oligomycin in desmin-negative cells from a UCMD and a BM patient. The graphs represent the response of the mitochondrial membrane potential after addition of oligomycin (indicated by arrows, Oligo) in desmin-negative cells from a UCMD (on left) and a BM (on right) patient, each trace represents the fluorescence values of a single cell. Where indicated by arrow 4 μ M FCCP (F) was added. A and A' are representative images of marked grid (see the numbers) used to identify cells previously analyzed in the TMRM study. B and B' are representative images of the immunohistochemical analysis, the myoblasts are marked with an antibody for desmin (red) and nuclei are stained by DAPI (blue).

We next identified desmin- and TUNEL-positive cells in the culture from one UCMD patient (Figure 26). We found that both myoblast and fibroblast populations from patients display a higher percentage of apoptotic cells than healthy donor cells.

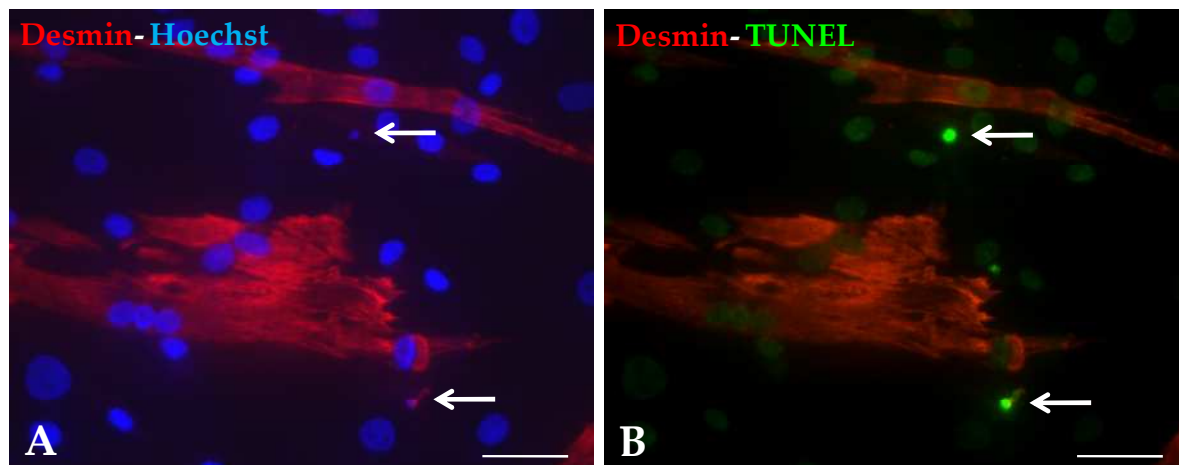


Figure 26. Desmin- and TUNEL-positive cells in cell cultures established from muscle biopsy of one UCMD patient. A and B are representative images of the immunohistochemical analysis performed on the cell cultures of a UCMD patient, the myoblasts are marked with an antibody for desmin (red), in Panel A all the nuclei are stained by Hoechst (blue) and in Panel B the apoptotic nuclei are marked by TUNEL reaction (green). Arrows underline two apoptotic nuclei: the upper of a desmin-negative cell and the lower of a myoblast. Bars, 50 μ m

These findings suggest that muscle fibroblasts display the pathological phenotype (mitochondrial dysfunction and high level of apoptosis) characteristic of patients affected by ColVI-related disorders. It should be noted that the percentage of desmin-positive cells

in primary muscle-derived cultures from patients was low from the beginning (just after 2 passages in culture it was usually less than 15%).

Lastly, we analyzed the oxygen consumption rate (OCR) of cells from UCMD and BM patients, to assess whether the mitochondrial respiration was different compared to a healthy donor. So far, we found that the basal respiration in patients was not significantly different from that of control (data not shown). However, encouraging results were obtained measuring the OCR in cells before and after the treatment with the SERCA inhibitor, thapsigargin. Even if further investigations are necessary, our preliminary data demonstrate that in healthy donor cells treatment for about 30 minutes with thapsigargin did not affect the OCR response to FCCP. On the other hand, in UCMD cells the treatment with thapsigargin prevented any stimulation by FCCP (Figure 27).

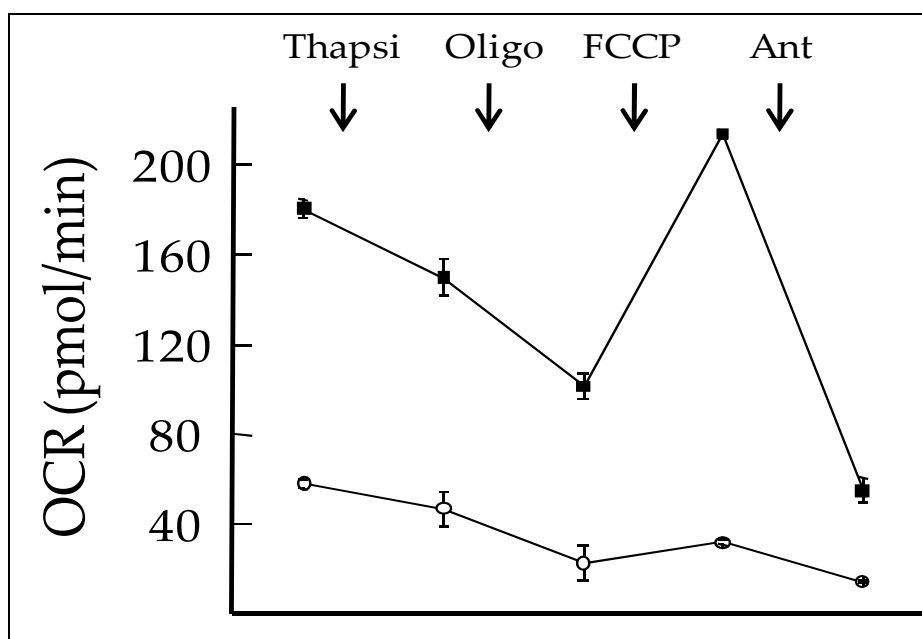


Figure 27. Mitochondrial respiration in cell cultures from a healthy donor and a UCMD patient.

The OCR was measured in intact cells as described in “Material and methods”. Where indicated by arrows 10 μ M thapsigargin (Thapsi), 0,3 μ M FCCP, 1,25 μ M oligomycin (Oligo) and 1,8 μ M antimycin (Ant) were added. The addition of FCCP to cells caused the increase of OCR in healthy donor (closed squares), but not in the UCMD patient (open circles). The data are the mean of three wells of cells \pm S.D.

As already suggested [4], our hypothesis is that thapsigargin causes an alteration of intracellular Ca^{2+} homeostasis affecting the PTP. The opening of the pore then would lead to loss of pyridine nucleotides [48;182], resulting in decrease of mitochondrial respiration. These findings further confirm that the mitochondrial dysfunction seen in patients affected by ColVI muscular dystrophies is latent, but also that proper stimuli can unmask the pathological phenotype.

CONCLUSIONS

The results presented in this Thesis provide compelling evidence that mitochondria are involved in the pathogenesis of ColVI muscular dystrophies; and they may represent a fundamental step towards a therapy of these diseases. A key role for mitochondria, and in particular for PTP, was proposed earlier, based on ultrastructural, functional and pharmacological data. However, the therapeutic effect of CsA was not yet a proof of concept that a cause-effect relationship between PTP inhibition and therapy of ColVI diseases exists, because of the multiple targets of this drug.

One of the goals of the present work was to obtain this proof of concept; Genetic ablation of CyP-D rescued the myopathic phenotype caused by ColVI deficiency, and had the same beneficial effect of CsA treatment.

Our work has also demonstrated that the non-immunosuppressive CyP inhibitor Debio 025 is an excellent candidate for the therapy of ColVI diseases, because is proved effective on the rescue of myopathic alterations yet does not expose the patients to the potential harmful effects of immunosuppression.

Finally, our study on muscle-derived cell cultures has confirmed that mitochondria of patients affected by both BM and UCMD display a latent dysfunction that can be revealed by treatment with oligomycin or rotenone, provided that the passage number is below 7. Thus, latent mitochondrial dysfunction appears to be a distinctive trait of all ColVI disorders, and one that can be exploited to evaluate the efficacy of experimental therapies.

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