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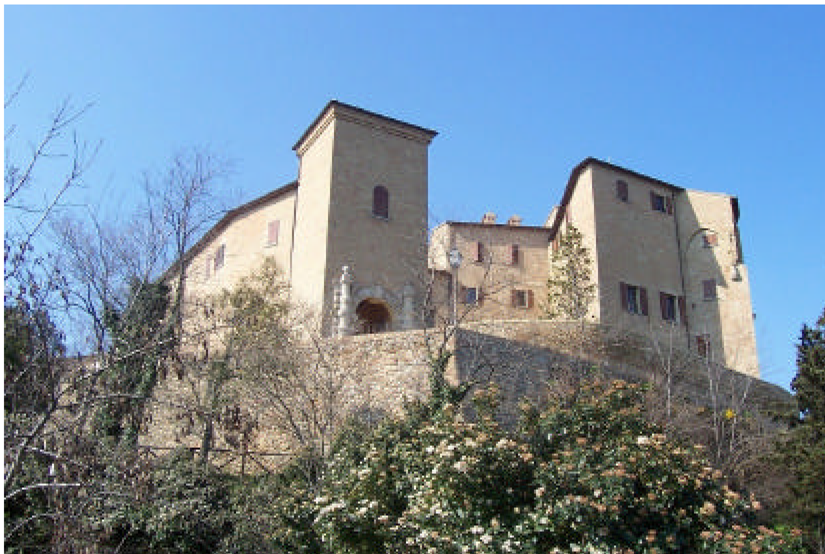
RIUNIONE ANNUALE 2010



Bertinoro 10-12 GIUGNO
Centro Residenziale Universitario, Rocca Vescovile, Bertinoro (Forlì)

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Gruppo Italiano di Biomembrane e Bioenergetica

Direttivo nazionale pro-tempore GIBB

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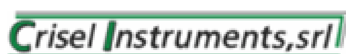

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PROGRAMME

THURSDAY 10th JUNE 2010

13:00 – 15:00	Registration
15:00 – 15:10	***Conference Opening*** ***P.Bernardi***
15:10 – 16:30	***MEMBRANES AND TRANSPORT*** ***Chairperson: P.Sarti***
15:10 – 15:30	PHOSPHOLIPASE ACTIVITIES IN GREEN COFFEE BEANS (COFFEA ARABICA L.) HARVESTED IN DIFFERENT COUNTRIES. Patui, S., Peresson, C., Clincon, L., Braidot, E., Zancani, M., Navarini, L., Colussi, A., Del Terra, L. and Vianello, A.
15:30– 15:50	EFFECT OF DETERGENTS ON THE ASSEMBLY OF THE ECTOPIC F_0F_1 ATP SYNTHASE IN RAT LIVER. Giovanna Lippe, Amit Kumar Rai, Valentina Giorgio, Elena Bisetto, Sabina Passamonti and Federica Dabbeni-Sala.
15:50– 16:10	PROBING THE BINDING SITE FOR ACYLCARNITINES OF THE MITOCHONDRIAL CARNITINE CARRIER BY SITE-DIRECTED MUTAGENESIS. Lara Console, Annamaria Tonazzi, Nicola Giangregorio, Cesare Indiveri, Ferdinando Palmieri.
16:10– 16:30	OVER-EXPRESSION AND PURIFICATION OF THE HUMAN OCTN1 AND OCTN2 TRANSPORTERS IN DETERGENT SOLUBLE FORM. Michele Galluccio, Lorena Pochini, Linda Amelio, Mariafrancesca Scalise, Cesare Indiveri
16:30 – 17:00	**Coffee break**
17:00 – 18:40	***MITOCHONDRIA IN CELL DEATH AND CANCER*** ***Chairperson: G.Lippe***
17.00 – 17:20	ANALYSIS OF POTENTIAL INVOLVEMENT OF MYTONIC DYSTROPHY PROTEIN KINASE IN DIFFERENT MITOCHONDRIAL CELL DEATH PATHWAYS. Boris Pantic, Elena Trevisan, Emanuela Berta, Paolo Bernardi, Sergio Salvatori and Andrea Rasola.
17:20 – 17:40	HOMOPLASMIC SHIFT OF *ND1* MUTATION REDUCES TUMOR GROWTH IN IMMUNODEFICIENT MICE. M. Capristo, G. Gasparre, I. Kurelac, A. Ghelli, G. Nicoletti, L. Lollini, M. Rugolo and A. M. Porcelli.
17:40 – 18:00	MITOCHONDRIAL ERK ACTIVATION AND THE ROLE CYCLOPHILIN D IN CANCER CELLS. Marco Sciacovelli, Federica Chiara, Boris Pantic, Giulia Guzzo, William S. Brusilow, Paolo Bernardi and Andrea Rasola.
18:00 – 18:20	MONOAMINE OXIDASE A AND B ARE MAJOR CONTRIBUTORS TO HEART FAILURE ONSET AND PROGRESSION. Nina Kaludercic, Eiki Takimoto, Takahiro Nagayama, Kevin Chen, Jean C Shih, Randy Blakely, David A Kass, Fabio Di Lisa, Nazareno Paolocci.
18:20 – 18:40	SINGLE POINT MUTATIONS OF A LYSINE RESIDUE CHANGE FUNCTION OF BAX AND BCL-XL EXPRESSED IN DKO MEF CELLS – NOVEL INSIGHTS INTO THE MOLECULAR MECHANISMS OF BAX-INDUCED APOPTOSIS. Luigi Leanza, Matthias Soddemann, Mario Zoratti, Erich Gulbins, Ildikò Szabò.
18:40 – 20:30	**Aperitivo di Benvenuto**

FRIDAY, 11th JUNE 2010

9:00 – 10:40 ***MITOCHONDRIAL PHYSIOLOGY AND PATHOLOGY I***
Chairperson: F.Dabbeni-Sala

9:00 – 9:20 MOLECULAR MECHANISMS OF MUSCLE-DERIVED STEM CELLS DIFFERENTIATION TO ADIPOCYTES: A NEW ROLE FOR P66SHC. Pallafacchina G., Zecchini E., Murgia M., Mammucari C., Raffaello A., Granatiero V., Rizzuto R.

9:20 – 9:40 BIOENERGETIC STUDIES OF FIBROBLASTS ISOLATED FROM HEALTHY CENTENARIANS. Gianluca Sgarbi, Marianna Del Sole, Alessandra Baracca, Catia Lanzarini, Stefano Salvioli, Andrea Cossarizza, Claudio Franceschi, Giorgio Lenaz, Giancarlo Solaini

9:40 – 10:00 REGULATION OF THE MITOCHONDRIAL PERMEABILITY TRANSITION PORE BY THE OUTER MEMBRANE TRANSLOCATOR PROTEIN (PERIPHERAL BENZODIAZEPINE RECEPTOR). Justina Šileikyte, Valeria Petronilli, Alessandra Zulian, Federica Dabbeni-Sala, Giuseppe Tognon, Peter Nikolov, Paolo Bernardi and Fernanda Ricchelli.

10:00 – 10:20 DEFINITION OF THE MITOCHONDRIAL CARRIER ALLOWING RAT-SELECTIVE ACTIVATION OF THE PERMEABILITY TRANSITION PORE BY NORBORMIDE. Alessandra Zulian, Valeria Petronilli, Sergio Bova, Federica Dabbeni-Sala, Gabriella Cargnelli, Paolo Bernardi and Fernanda Ricchelli.

10:20 – 10:40 ASSEMBLY AND STABILITY OF RESPIRATORY CHAIN SUPERCOMPLEXES IS AFFECTED BY REMODELING OF MITOCHONDRIAL CRISTAE: FOCUS ON OPA1. Sara Cogliati, Christian Frezza, Patricio Fernandez Silva, Antonio Josè Enriquez, Luca Scorrano.

10:40 – 10:50 STOP GOING IN CYCLES…USE MACS TECHNOLOGY FOR MITOCHONDRIA ISOLATION. Miltenyi-Biotec.

10:50 – 11:10 **Coffee break**

11:10 – 12:30 ***RNS AND ROS BIOLOGY***
Chairperson: C.Indiveri

11:10 – 11:30 REDOX PROPERTIES OF NOVEL MITOCHONDRION-TARGETED QUERCETIN AND RESVERATROL DERIVATIVES. Mattarei Andrea, Sassi Nicola, Marotta Ester, Durante Christian, Gennaro Armando, Paradisi Cristina, Zoratti Mario, Biasutto Lucia.

11:30 – 11:50 IN VITRO TOXICOLOGY OF MITOCHONDRIOTROPIC POLYPHENOLS. Sassi Nicola, Biasutto Lucia, Mattarei Andrea, Marotta Ester, Paradisi Cristina, Zoratti Mario.

11:50 – 12:10 FLAVOHEMOGLOBIN AND NO-DETOXIFICATION IN THE HUMAN PROTOZOAN PARASITE *GIARDIA INTESTINALIS.* Alessandro Giuffrè, Daniela Mastronicola, Fabrizio Testa, Elena Forte, Marzia Arese, Eugenio Bordi, Leopoldo Paolo Pucillo and Paolo Sarti.

12:10 – 12:30 HCV INFECTION CAUSES ENHANCED MITOCHONDRIAL CA^{2+}-SENSITIVE LIPID ACCUMULATION UP-REGULATING THE ACETYL COA CARBOXYLASE ACTIVITY. Ripoli M, Piccoli C, D'Aprile A, Pazienza V, Moradpour D, Capitanio N.

12:30 **LUNCH**

14:15 – 16:00 ***MOLECULAR STRUCTURE AND FUNCTION OF MEMBRANE PROTEINS***
Chairperson: N.Capitanio

14:15 – 15:00 **GUEST LECTURE:** STRUCTURE AND EVOLUTION OF MITOCHONDRIAL OUTER MEMBRANE PROTEINS **Cornelius Zeth.**

15:00 – 15:20 IS THE N-TERMINAL SEQUENCE IMPORTANT FOR THE FUNCTIONALITY OF THE VOLTAGE DEPENDENT ANION CHANNEL (VDAC)? Simona Reina, Angela Messina, Francesca Guarino, Flora Tomasello and Vito De Pinto.

15:20 – 15:40 INTRINSIC UNCOUPLING: ADP AND PI MODULATION IN THE WT AND ε-TRUNCATED ATP SYNTHASES OF *ESCHERICHIA COLI.* D'Alessandro M., Turina P., Dunn. S.D., Melandri B.A.

15:40 – 16:00 THE ROLE OF BOUND WATER MOLECULES IN STABILIZING THE PRIMARY CHARGE SEPARATION OF PHOTOSYNTHETIC REACTION CENTERS. Marco Malferrari, Francesco Francia, Giovanni Venturoli.

16:00 – 16:30 **Coffe break**

16:30 – 18:30 ***SESSION IN HONOUR OF ANDREA MELANDRI***
Chairperson: D.Zannoni

16:30 – 16:45 Davide Zannoni

16:45 – 17:30 **GUEST LECTURE:** "PHOTOSYSTEM I - FROM STRUCTURE TO PHOTOELECTRIC DEVICES" **Nathan Nelson**

17:30 –18:30 Contributions of Sergio Papa, Paolo Pupillo, Giorgio Forti, Giorgio Lenaz

19:00 – 20:00 **Meeting for GIBB members**

20:30 **SOCIAL DINNER**

SATURDAY, 12th JUNE 2010

9:30 – 11:30 ***MITOCHONDRIAL PHYSIOLOGY AND PATHOLOGY II***
Chairperson: G.Solaini

9:30 – 9:50 BIOCHEMICAL CHARACTERIZATION ON POLYPEPTIDES OF HUMAN MRP6 PROTEIN. Rocchina Miglionico, Angela Ostuni, Maria Antonietta Castiglione Morelli and Faustino Bisaccia.

9:50 – 10:10 MODULATION OF THE MITOCHONDRIAL PERMEABILITY TRANSITION BY QUINONES – THE CASE OF IDEBENONE. Valentina Giorgio, Valeria Petronilli, Maurizio Prato, Anna Maria Ghelli, Michela Rugolo and Paolo Bernardi.

10:10 – 10:30 MOLECULAR INVESTIGATION OF RIBOFLAVIN-RESPONSIVE MULTIPLE ACYL-COA DEHYDROGENASE DEFICIENCY (RR-MAD) PATIENTS. Adriana Malena, Annalisa Botta, Maria C. Bellocchi, Gessica Smaniotto, Ivano Eberini, Cristina Sensi, Elena Pgoraro, Emanuele Loro, Lodovica Vergani.

10:30 – 10:50 MITOCHONDRIAL BIOGENESIS INDUCED BY PPARS AGONISTS IMPROVES VIABILITY OF LEBER'S HEREDITARY OPTIC NEUROPATHY (LHON) CYBRIDS. Luisa Iommarini, Michela Rugolo, Carlos T. Moraes, Valerio Carelli.

10:50 – 11:10 TOWARD A MITOCHONDRIAL THERAPY FOR COLLAGEN VI MUSCULAR DYSTROPHIES. Elena Palma, Tania Tiepolo, Alessia Angelin, Patrizia Sabatelli, Luciano Merlini, Luca Nicolosi, Francesca Finetti, Paola Braghetta, Grégoire Vuagniaux, Jean-Maurice Dumont, Cosima Tatiana Baldari, Paolo Bonaldo and Paolo Bernardi.

11:10 – 11:30 A CA^{2+}-REGULATED MITOCHONDRIAL (PERMEABILITY TRANSITION) PORE IN *DROSOPHILA MELANOGASTER*. Sophia von Stockum, Emy Basso, Valeria Petronilli, Mike Forte and Paolo Bernardi.

11:30 – 12:00 **Coffee break**

12:00 – 12:10 Award of Assunta Baccarini-Melandri Prize.

12:10 – 12:25 ***Conference Closing***
B.A.Melandri

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MEMBRANES AND TRANSPORT

PHOSPHOLIPASE ACTIVITIES IN GREEN COFFEE BEANS (COFFEA ARABICA L.) HARVESTED IN DIFFERENT COUNTRIES

Patui, S.*, Peresson, C.*, Clincon, L.*, Braidot, E.*, Zancani, M.*, Navarini, L.§, Colussi, A.§, Del Terra, L.§ and Vianello, A.*

**Sezione di Biologia Vegetale, Dipartimento Biologia e Protezione delle Piante, Università di Udine, Via delle Scienze, 91, I-33100 Udine, Italy. §Research and Innovation, Illycaffè, Biolab, via Flavia 110, I-34147 Trieste, Italy*

Triacylglycerols (TAGs) are accumulated in specialised organelles called "oil bodies", which are enclosed in a phospholipid monolayer embedded with some unique proteins. Upon germination, such membranes are modified to allow the availability of TAGs as an energy source during early stages of seedling growth in oilseeds. This process occurs by the sequential and/or collective action of many hydrolytic enzymes, such as phospholipases, lipoxygenases and lipases that are associated to oil body membranes. In contrast, during seed storage, oilseed lipids may undergo lipolytic degradation processes leading to a wide range of metabolites potentially harmful for seed viability.

In particular, green coffee endosperm consist of approx. 99% of the mature seed mass and contains many polyunsaturated fatty acids whose degradation leads to volatile compound formation through the oxylipin pathway. In spite of this, the enzymes involved in TAGs degradation (particularly lipases) are poorly studied. Therefore, the aim of this work was to evaluate the involvement of phospholipase activity in oil body membrane degradation during storage of green coffee (*Coffea arabica* L.) and to determine the correlations between storage lipid mobilization and maintenance of seed viability in beans harvested in different countries (Ethiopia, India, Kenya and Tanzania). Green coffee beans were frozen and powdered in liquid nitrogen and oil bodies were extracted with cold acetone. Phospholipase A_1 and A_2 activities were assayed in crude extracts by a fluorimetric method, using different probes. Such activities were just partially stimulated by free Ca^{2+}, in contrast with what reported by others. Furthermore, PLA_2 activity was assayed in a wide range of pH, evidencing two peaks of pH optimum. These results suggests that green coffee bean presents at least two isoforms of PLA_2. Phospholipase profiles (PLA_2 and total) were correlated with the provenience of the beans, showing a higher activity in those harvested in Ethiopia, while the lower was associated to beans from India.

EFFECT OF DETERGENTS ON THE ASSEMBLY OF THE ECTOPIC F_OF_1 ATP SYNTHASE IN RAT LIVER

Giovanna Lippe°, Amit Kumar Rai*, Valentina Giorgio#, Elena Bisetto#, Sabina Passamonti^ and Federica Dabbeni-Sala*

*°Department of Food Science and #Department of Biomedical Sciences and Technologies, University of Udine, ^Department of Life Sciences, University of Trieste, *Department of Pharmacology, University of Padova*

Several studies have established that the F_0F_1ATP synthase complex localized on cell surface (ecto-F_OF_1) of hepatocytes plays a role in the uptake of HDL-bound cholesterol, along with the well characterized scavenger receptor-BI (SR-BI). Binding of HDL via apo-AI to the ? chain of ecto-F_OF_1, which has the F_1 moiety facing outside, is believed to trigger extracellular ATP hydrolysis and, consequently, ADP-dependent activation of P2Y13, a G protein-coupled purinergic receptor participating in HDL endocytosis (1).

In accordance with the role of ecto-F_OF_1 in HDL metabolism, we recently found that in animals where the bile duct has been ligated, to induce a short-term extra-hepatic cholestasis, the ectopic complex is down-regulated in the liver. In fact, this condition was characterized by high levels of cholesterol in the liver, variations in HDL levels in the plasma and down-regulation of SR-BI. In particular, we found that in cholestatic animals the ecto-F_OF_1 ATPase activity was stably reduced, as a consequence of an increased binding of IF_1 to ecto-F_OF_1. Because no activity change of the mitochondrial F_OF_1 ATP synthase, or any variation of its association with IF_1 was observed in cholestatic rat mitochondria, our result indicated that ecto-IF_1 expression level was modulated independently from that of ecto-F_OF_1,and the mitochondrial IF_1 and F_OF_1 (2).

The aim of the present work was to set up the conditions to partially purify ecto-F_OF_1 from plasma membrane preparations of liver from control and pathologic rats, in order to define the assembly of the complex. We performed extraction with different mild detergents, i.e. dodecylmaltoside, Triton or digitonin, followed by BN- and hrCN- PAGEs separation, where the presence of F_OF_1 complexes was established by in-gel ATPase activity staining and immunoblotting. All treatments evidenced that plasma membranes contain low amounts of F_OF_1 complexes displaying a similar molecular weight to the monomeric form of the mitochondrial F_OF_1 ATP synthase. This indicates that the plasma membranes do contain complete, functional F_OF_1 ATP synthase complexes, which are stable to treatment with different detergents and display very similar assembly of the mitochondrial enzymes.

1. Jacquet, S.et al. (2005). Cell Mol Life Sci 62, 2508-2515.
2. Giorgio, V. et al. (2010) J Bioenerg Biomembr. 42(2):117-23.

AKR is supported by a fellowship from Fondazione Cassa di Risparmio di Padova e Rovigo.

PROBING THE BINDING SITE FOR ACYLCARNITINES OF THE MITOCHONDRIAL CARNITINE CARRIER BY SITE-DIRECTED MUTAGENESIS

Lara Console[a], Annamaria Tonazzi[a,b], Nicola Giangregorio[a,b], Cesare Indiveri[c], Ferdinando Palmieri[a,b]

[a]Department of Pharmaco-Biology, Laboratory of Biochemistry and Molecular Biology, University of Bari, 70125 Bari, Italy; [b]CNR Institute of Biomembranes and Bioenergetics, via Orabona 4,70125 Bari, Italy; [c]Department of Cellular Biology, University of Calabria, 87036 Arcavacata di Rende, Italy

The carnitine/acylcarnitine carrier (CAC) belongs to the mitochondrial carrier family. It catalyzes the transport of acylcarnitines into the mitochondrial matrix in exchange with free carnitine. The sequence conservation in the mitochondrial carrier family suggests that the main structural fold is similar for all carriers. Indeed the CAC structure was constructed by homology modelling on the basis of the sole available ADP/ATP carrier structure. It consists of six transmembrane a-helices which surround a central water filled cavity opened towards the cytosol and closed toward the matrix by a network of six charged amino acids forming salt bridges. Among these residues, K35 and E132, play a major role in opening/closing the matrix gate. R275 and D179 which protrude into the cavity about the midpoint of the membrane, bind carnitine. However, since CAC transports also acylcarnitines, the identification of residues which bind the acyl moieties is an important point to define the molecular mechanism of transport. We have found that hydrophobic amino acid residues facing the cavity are grouped in a half-shell shaped surface, corresponding to the adjacent a–helices I, II and VI. The role of residues of a–helices I and II in the acylcarnitine binding has been investigated by site-directed mutagenesis of the hydrophobic amino acids facing the cavity. Acylcarnitines are competitive inhibitors of carnitine. Thus the interaction with the transporter can be evaluated using acylcarnitines (not available in radioactive forms) as inhibitors of ^{3}H-carnitine/carnitine transport. We have studied the effect of substitution of amino acid residues with Ala, on the sensitivity towards acylcarnitines from 2 to 16 carbon chains, as percent loss inhibition respect to the WT. Previous data showed that H29 is involved in the acylcarnitine binding, since it forms an H-bond with the ß-O- of acylcarnitines. Besides this residue, L14, G17, G21, V25, P78, V82, M85, F93 and C89 have been mutated. Most of the mutants showed transport function measured as [^{3}H] carnitine/carnitine antiport ranging from 30 to 100 % of the WT with the exception of G17A which was nearly inactive. The percent of inhibition loss respect to WT was tested for the various acylcarnitines. L14A, G21A and F93A did not cause significant variations of inhibition by acylcarnitines. Whereas, V25A, H29A, P78A and M85A mutations caused loss of inhibition of acylcarnitines from 2 to 14 C, more evident for the medium chain length (C8-C12) in the case of V25A, H29A and M85A. V82A and C89A caused a loss of inhibition of all the acylcarnitines. The experimental results allowed us to draw a map of the interaction between acylcarnitines and the active site of CAC which are supported by docking simulations.

OVER-EXPRESSION AND PURIFICATION OF THE HUMAN OCTN1 AND OCTN2 TRANSPORTERS IN DETERGENT SOLUBLE FORM

Michele Galluccio, Lorena Pochini, Linda Amelio, Mariafrancesca Scalise, Cesare Indiveri

Dipartimento di Biologia Cellulare, Università della Calabria, via P.Bucci 4c 87036 Arcavacata di Rende (CS)

The transporter OCTN1 and OCTN2 are members of the OCTN (organic cation transporters novel) sub family. These transporters are involved in the carnitine homeostasis. So far, only OCTN2 has been demonstrated to catalyse carnitine transport [1 and refs. herein]. The function of OCTN1 is still unclear [see 2 for refs.]. The defect of OCTN1 or OCTN2 are associated with severe pathologies such as the primary carnitine deficiency, Crohn disease and ulcerative colitis. We have reconstituted OCTN2 extracted from kidney in proteoliposomes. It catalyzes a sodium-dependent antiport of carnitine with carnitine derivatives. We also showed that omeprazole interact with SH groups of the transporter, inactivating the transport function. Recently we have over-expressed human OCTN1 and purified the protein by affinity chromatography [2].
In this work we have pointed out a first procedure for over-expression of the human OCTN2 and improved the purification of OCTN1 for crystallography purposes.
The cDNA coding for OCTN2 protein, was cloned in several expression plasmids, but without success. Only upon insertion of the cDNA in pET41a+ (pET41a+-OCTN2) coding for a GST-OCTN2 fusion protein, a low level expression was obtained in E.coli Rosetta DE3 cells. The expression was largely improved using TY medium for cell growth under high osmolarity conditions (1M Sorbitol). The over-expressed protein was collected as insoluble fraction of the cell lysate. For functional and structural studies the protein needed to be refolded and solubilized. To this aim the protein was solubilized in urea and sarkosyl and then the detergent was changed by elution on Profinity IMAC Ni-charged column in the presence of Triton X-100. The eluted protein was Triton soluble and, after treatment with thrombin, it was separated from the GST tag by gel filtration chromatography. Further attempt to over-express the untagged protein are in course by changing the E. coli strains, growth conditions and other parameters.
Concerning the OCTN1, in order to perform structural studies, we have optimized a large scale purification and refolding in LDAO of the previously expressed protein. Using a Hi-Trap column eluted with a gradient of imidazole from 0 to 500 mM, a purified protein fraction more than 99% pure was obtained with 100 mM imidazole. The protein yield was 1 mg of purified LDAO soluble OCTN1 per liter of cell culture.

1. L. Pochini, F. Oppedisano, C. Indiveri, Reconstitution into liposomes and functional characterization of the carnitine transporter from renal cell plasma membrane, Biochim. Biophys. Acta 1661 (2004) 78-86.
2. Galluccio M, Pochini L, Amelio L, Accardi R, Tommasino M, Indiveri C. Over-expression in E. coli and purification of the human OCTN1 transport protein, Protein Expr Purif. 68 (2009) 215-20.

MITOCHONDRIA IN CELL DEATH AND CANCER

ANALYSIS OF POTENTIAL INVOLVEMENT OF MYTONIC DYSTROPHY PROTEIN KINASE IN DIFFERENT MITOCHONDRIAL CELL DEATH PATHWAYS

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Myotonic dystrophy1 (DM1) is a multi-system, autosomal dominant disorder which results from a CTG repeat expansion located in the 3' untranslated region of *DMPK* gene coding for a Ser/Thr kinase. Mouse models have demonstrated that decreased levels of *DMPK*, Six5 (the *DMPK*-5' flanking gene) and the expression of expanded CUG repeats independently contribute to the development of DM1 pathology. The *DMPK* protein is ubiquitously expressed and its biological functions are poorly understood. Among six major *DMPK* isoforms, the human isoforms A and C have been shown to localize to the outer mitochondrial membrane by their hydrophobic C-terminal tails. Here we investigate the effects of the expression of human *DMPK* isoform A in the SAOS-2 osteosarcoma cell model lacking the endogenous kinase. Preliminary results indicate that *DMPK* expression alters susceptibility of SAOS-2 cells to death stimuli such as detachment of hexokinase II from mitochondria, oxidative stress and nutrient depletion. Hexokinase detachment and oxidative stress caused by diamide are well-known inducers of the mitochondrial permeability transition pore (PTP), thus opening the possibility of a new and yet undefined regulatory mechanism functionally linking mitochondria-anchored *DMPK* isoform A to the PTP.

HOMOPLASMIC SHIFT OF ND1 MUTATION REDUCES TUMOR GROWTH IN IMMUNODEFICIENT MICE

M. Capristo[1], G. Gasparre[2], I. Kurelac[2], A. Ghelli[1], G. Nicoletti[3], L. Lollini[3], M. Rugolo[2] and A. M. Porcelli[2]

[1]Department of Biology, [2]Department of Gynecological, Obstetric and Pediatric Sciences, Medical Genetics, [3]Cancer Research Section, Department of Experimental Pathology, University of Bologna, Bologna, Italy

Mutations in mitochondrial DNA (mtDNA) have been described in several types of tumors, however, the relationship between mtDNA mutations and tumor development has been seldom analyzed [1-4]. A few studies reported the induction of a higher tumorigenic and invasive potential in cybrid cells harbouring mtDNA mutations [2, 5, 6], suggesting that such mutations may confer a growth advantage to transformed cells. Since mtDNA exists in many copies in a cell, mtDNA mutations can be homoplasmic (all copies are identical) and heteroplasmic (two or more variants coexist). Although mtDNA mutations found in tumors are often homoplasmic, it has been recently reported that heteroplasmic mutations only promote tumorigenesis [3]. To define the impact of homoplasmic and heteroplasmic mtDNA mutations on tumor development and progression, we inoculated cybrid cell lines carrying either homoplasmic or heteroplasmic insertion in the ND1 gene into nude mice. The xenograft masses were considerably larger in mice inoculated with heteroplasmic cells, except in one animal, that developed a tumor comparable to those induced by homoplasmic cells. Noticeably, in this tumor a significantly higher ND1 mutant load was detected. The same result was obtained in cell cultures derived from this xenograft mass, suggesting that the homoplasmic shift could be preserved in vitro. We have taken advantage of this cell line to analyze the effect of the mutational shift on the energetic competence and metabolic adaptation of cancer cells, in particular evaluating complex I assembly/function and stabilization of hypoxia-inducible factor 1 (HIF1). This is the first evidence for a homoplasmic shift of a mtDNA mutation occurring in vivo in a tumor. This finding allows us to demonstrate its potential advantageous effect on counteracting tumor progression. The analysis is ongoing and the final results will be presented.

[1] G. Gasparre et al., Proc Natl Acad Sci U S A 104 (2007) 9001-6.
[2] K. Ishikawa et al., Science 320 (2008) 661-4.
[3] J.S. Park et al., Hum Mol Genet 18 (2009) 1578-89.
[4] A.M. Porcelli et al., Hum Mol Genet 19 (2010) 1019-32.
[5] Y. Shidara et al., Cancer Res 65 (2005) 1655-63.
[6] J.A. Petros et al., Proc Natl Acad Sci U S A 102 (2005) 719-24

MITOCHONDRIAL ERK ACTIVATION AND THE ROLE OF CYCLOPHILIN D IN CANCER CELLS

Marco Sciacovelli, Federica Chiara, Boris Pantic, Giulia Guzzo, William S. Brusilow[a], Paolo Bernardi and Andrea Rasola

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We have studied human cancer cell models where we detected constitutive activation of ERK. A fraction of active ERK was found to be located in mitochondria in RWPE-2 cells, obtained by Ki-Ras transformation of the epithelial prostate RWPE-1 cell line; and in osteosarcoma SAOS-2 cells. All these tumor cells displayed marked resistance to death caused by apoptotic stimuli like arachidonic acid and the BH3 mimetic EM20-25, which are mitochondrial permeability transition pore (PTP) inducers. This marked resistance to cell death could be ablated by inhibiting ERK with PD98059 or with a selective inhibitor peptide (EIP). ERK inhibition enhanced GSK-3-dependent phosphorylation in Ser/Thr residues of the pore regulator Cyclophilin D, whereas GSK-3 inhibition protected from PTP opening. Thus, in tumor cells mitochondrial ERK activation desensitizes the PTP through a signalling axis that involves GSK-3 and Cyclophilin D, a finding that provides a mechanistic basis for increased resistance to apoptosis of neoplastic cells.

MONOAMINE OXIDASE A AND B ARE MAJOR CONTRIBUTORS TO HEART FAILURE ONSET AND PROGRESSION

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Monoamine oxidases A and B (MAO-A, -B) are mitochondrial enzymes deputed to degradation of catecholamines (CA) such as norepinephrine (NE) or dopamine (DA) and their activity results in the production of hydrogen peroxide. Considering that oxidative stress and CA spillover into the plasma are hallmarks of congestive heart failure (CHF), MAOs are well suited to play a major role in this syndrome. To this aim, WT, dominant negative MAO-A (MAO-Aneo) and MAO-B knockout mice (MAO-B-/-) were subjected to CHF via transverse aortic constriction (TAC, 9 weeks). Pressure-volume (PV) loops analysis showed that after 9 weeks of TAC WT hearts had increased left ventricle (LV) dimensions and impaired function. Conversely, MAO-Aneo and MAO-B-/- mice showed preserved end-systolic (ESV) and end-diastolic (EDV) volumes and improved LV function: ESV was 5.5±1.4 in MAO-Aneo and 5.5±0.8 in MAO-B-/- vs 16.6±4.5 µl in WT and ejection fraction was 79.3±3 in MAO-Aneo and 79±1 in MAO-B-/- vs 59±8% in WT (both $p<0.01$). NE and DA content was depleted in WT but not in MAO-Aneo and MAO-B-/- hearts, suggesting that CA catabolism is increased in failing hearts resulting in higher MAO activity. WT hearts also showed increased fibrosis after TAC that was reduced in MAO-Aneo but not in MAO-B-/- mice, suggesting that the two isoforms mediate separate signaling pathways. In conclusion, MAO activity plays a central role in controlling cardiac function and when inhibited prevents LV remodeling, thus sustaining inotropy in failing hearts.

SINGLE POINT MUTATIONS OF A LYSINE RESIDUE CHANGE FUNCTION OF BAX AND BCL-XL EXPRESSED IN DKO MEF CELLS – NOVEL INSIGHTS INTO THE MOLECULAR MECHANISMS OF BAX-INDUCED APOPTOSIS

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Members of the Bcl-2 family play key roles as proapoptotic (e.g. Bax) and antiapoptotic (e.g. Bcl-xL) regulators of programmed cell death. We previously identified a mitochondrial potassium channel Kv1.3 as a novel target of Bax. Incubating Kv1.3-positive isolated mitochondria with Bax triggered apoptotic events whereas Kv1.3-deficient mitochondria were resistant to this stimulus. Mutation of Bax at lysine 128 (BaxK128E) abrogated its effects on Kv1.3 and mitochondria. These data indicate a toxin-like action of Bax on Kv1.3 to trigger at least some of the mitochondrial changes typical for apoptosis. To gain insight into the mechanism, we mutated Glu158 of Bcl-xL (corresponding to K128 in Bax) to lysine. This substitution turned Bcl-xL proapoptotic.
Transfection of double knock-out (Bax-/- Bak-/-) mouse embryonic fibroblasts (DKO MEF) with either wild-type Bax, Bax(K128E), or Bcl-xL(E158K) showed that staurosporine induced apoptosis was defective in DKO MEF and Bax(K128E)-transfected cells, but was recovered upon transfection with Bcl-xL(E158K) or Bax. Both wild-type Bax and Bax K128E can form similar ion-conducting pores upon incorporation into planar lipid bilayers. Our results point to a physiologically relevant interaction of Bax with Kv1.3 and further confirm the role of a crucial lysine in determining the proapoptotic character of Bcl2-family proteins.

MITOCHONDRIAL PHYSIOLOGY AND PATHOLOGY I

MOLECULAR MECHANISMS OF MUSCLE-DERIVED STEM CELLS DIFFERENTIATION TO ADIPOCYTES: A NEW ROLE FOR P66SHC

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Shc adaptor proteins are normally cytosolic and participate in the transduction of growth factor signals. There are three Shc isoforms, p46, p52 and p66. The latter sensitizes cells to apoptosis, promotes aging and is involved in cell stress responses downstream of PKCbeta. PKCbeta phosphorylates p66 following oxidative stress, leading to its translocation to mitochondria. Once inside the mitochondria, p66 acts as oxidoreductase by transferring electrons from cytochrome c to molecular oxygen, thus producing reactive oxygen species (ROS).

Recently, our laboratory demonstrated that primary culture of skeletal muscle derived stem cells (MDSCs), which consists mainly in myogenic cells that differentiate into myotubes when switched to low serum medium, are able to transdifferentiate into adipocytes when cultivated in a high glucose medium, condition that mimics the hyperglycemic environment. PKCbeta mediates this transdifferentiation, since treatment of cells with high glucose medium rapidly induces PKCbeta translocation to plasma membrane and its activation, while silencing PKCbeta by RNAi dramatically reduces the adipocytic differentiation of MDSCs. The pro-adipogenic action of PKCbeta on MDSCs depends on its capacity to induce ROS, since treatment of cells with H_2O_2 was as effective as high glucose in promoting adipocytic differentiation.

We are presently investigating the role of p66, as the target of PKCbeta-mediated ROS production, in the induction of MDSCs trandifferentiation. We compared MDSCs from wt and p66(-/-) mice and observed their capacity to differentiate into adipocytes in response to high glucose. MDSCs from p66(-/-) show a dramatic reduction in the ability to differentiate into mature adipocytes. In addition we challenged wt and p66(-/-) muscle tissue in vivo with oxidative (injection of H2O2), and pro-adipogenic stress (injection of glycerol) and analysed the activation of apoptotic/autophagic response and the induction of adipogenesis, respectively. p66(-/-) muscles show a dramatic reduction of adipogenesis and a decreased response to oxidative stress, accompanied by a strong induction of the autophagy response in vivo, compared to wt tissue. To demonstrate that the adipogenic effect of PKCbeta/p66/ROS signaling involves alteration of mitochondria physiology, we are also investigating the role of p66 in the regulation of mitochondrial calcium (Ca^{2+}) dynamics in MDSCs. We are measuring agonist-dependent changes in mitochondrial Ca^{2+} concentration via the transfection of targeted luminescent probes. We previously showed that p66(-/-) MEFs upon oxidative stress display alteration of mitochondrial Ca^{2+} signaling, however, to date no data are available for MDSCs primary cells. Our work would provide new insights to the complex molecular mechanisms responsible for the transdifferentiation of myogenic cells into adipocytes in response to hyperglycemia/oxidative stress, which start from the extracellular oxidative stress, going through PKCbeta activation, p66 translocation, ROS production, alteration of mitochondria Ca^{2+} homeostasis and, finally, expression of adipogenic genes.

BIOENERGETIC STUDIES OF FIBROBLASTS ISOLATED FROM HEALTHY CENTENARIANS

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According to a widely accepted theory, the mitochondrial theory of ageing (Linnane et al. 1989), electron transport through the respiratory chain generate reactive oxygen species (ROS), which in turn damage respiratory chain complexes and mitochondrial DNA (mtDNA). This will determine further ROS production that will increase mtDNA damage and so on. The result is a progressive damage of cells and tissues that is seen at the basis of the ageing process. Centenarians are exceptionally long living individuals who escaped the most common age-related diseases. In particular, they appear to be effectively protected from many degenerative disorders, including neurodegeneration, diabetes, cardiovascular diseases, tumours, that are all pathological conditions in which mitochondria are believed to play a crucial role at least in their progression (Wallace 2005). The mechanisms that underlie the particularly good condition of the centenarians health are still largely unclear. To critically analyse the possible mitochondrial contribution to the healthy tissues in centenarians, we investigated bioenergetic parameters of fibroblasts of four centenarians (96-102 years). We assayed ATP synthesis rate, intracellular ATP content, mitochondrial mass and structural organization of OXPHOS complexes and compared the results with data obtained from fibroblasts of three young individuals (25-30 years), taken as controls. Fibroblasts were obtained from dermal biopsies. Cells of centenarians appear characterised by a higher protein/10^6 cells ratio and an increased content of mitochondria, as measured by citrate synthase activity (an index of mitochondrial mass). Concurrently, fibroblasts of centenarians showed a marked increase in the intracellular ATP content and a slight increase in the rate of ATP synthesis, using glutamate/malate as substrates. Normalized to citrate synthase activity, the results showed that mitochondrial function is hardly affected in centenarians compared to controls. In agreement with the mitochondrial mass, 2D electrophoretic analysis of OXPHOS complexes showed a higher amount of complex I, IV and V in fibroblasts of centenarians compared to controls. Moreover, the molecular organization of OXPHOS complexes in centenarians' fibroblasts showed a higher aggregation level of super-complex I-III. These findings will be discussed in relation with the intracellular ROS content, that is currently under investigation.

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REGULATION OF THE MITOCHONDRIAL PERMEABILITY TRANSITION PORE BY THE OUTER MEMBRANE TRANSLOCATOR PROTEIN (PERIPHERAL BENZODIAZEPINE RECEPTOR)

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We have studied the properties of the permeability transition pore (PTP) in rat liver mitochondria and in mitoplasts retaining inner membrane ultrastructure and energy-linked functions. Mitoplasts readily underwent a permeability transition following Ca^{2+} uptake, in a process that maintained sensitivity to cyclosporin A. On the other hand, interesting differences between mitochondria and mitoplasts emerged in PTP regulation by redox events. Photoirradiation of hematoporphyrin (HP)-loaded mitochondria inhibits the PTP through photosensitization of matrix His residues [Salet et al. (1997) *J. Biol. Chem.* 272, 21938-21943]. PTP reactivation can then be achieved with higher light doses, an effect that is specific for dicarboxylic porphyrins endowed with protoporphyrin IX (PP) configuration like HP, deuteroporphyrin and PP itself, which bind the outer membrane translocator protein-18 kDa (TSPO, formerly known as peripheral benzodiazepine receptor). We show that HP-loaded mitoplasts underwent pore inactivation through photosensitization of matrix His but were totally resistant to PTP reactivation, indicating that the outer membrane is specifically required for the latter process to occur. PTP reactivation in mitochondria was selectively antagonized by N,N-dihexyl-2-(4-fluorophenyl)indole-3 acetamide, a porphyrin-competitive specific ligand of TSPO. These results indicate that the site mediating PTP reactivation is located in the outer membrane, and suggest that it involves TSPO. Thus, the permeability transition is an inner membrane event, yet it can be regulated by the outer membrane through specific interactions with TSPO.

DEFINITION OF THE MITOCHONDRIAL CARRIER ALLOWING RAT-SELECTIVE ACTIVATION OF THE PERMEABILITY TRANSITION PORE BY NORBORMIDE

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The rat-selective toxicant norbormide (NRB) induces opening of the mitochondrial permeability transition (PT) pore (PTP) in rats but not in mice or guinea pigs. The key structural feature of NRB responsible for PTP activation is the (phenylvinyl)pyridine subunit (DR166), which favors PTP opening following structural perturbations of inner mitochondrial membrane (IMM) lipid domains. Selectivity towards the rat PT is probably due to the unique properties of a surface transport system allowing drug internalisation in rat mitochondria. To identify this putative NRB carrier we investigated the influence of the drug on the reactivity of a class of sulfhydryls which regulate the PTP from sites located on the outer surface of the inner membrane. For comparison, the influence of NRB on the reactivity of internal, PTP-regulating thiols was also studied. As external thiols reside in PTP domains which are strictly associated to regions of the translocator protein (TSPO, formerly known as the peripheral benzodiazepine receptor) specific to dicarboxylic porphyrins endowed with protoporphyrin (PP)-like configuration, the NRB effects were also tested in the presence of PP-like and PP-unrelated porphyrins. We show that NRB stimulates the PTP-regulatory activity of both internal and external thiols; in addition, it modifies the conformation of the external thiol binding sites during mitochondrial internalisation. TSPO-bound dicarboxylic porphyrins specifically antagonize the stimulatory effect of NRB on the PT, hindering drug translocation into internal PTP domains. None of these effects was observed in mouse and guinea pig mitochondria. We conclude that: (i) NRB transport into rat mitochondria occurs via a carrier associated to or comprising TSPO domains; (ii) species-selectivity of NRB towards the PT can be ascribed to a unique structure of these latter domains in rat mitochondria.

ASSEMBLY AND STABILITY OF RESPIRATORY CHAIN SUPERCOMPLEXES IS AFFECTED BY REMODELING OF MITOCHONDRIAL CRISTAE: FOCUS ON OPA1

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Mitochondria are central organelles in metabolism, signal transduction and programmed cell death. Their structure is extremely complex, being the organelle bound by two membranes. The inner membrane (IMM) is further organized in two distinct compartments, the so called "boundary membrane" and the cristae, separated from the former by narrow tubular junctions. We have recently shown that the pro-fusion dynamin-like protein optic atrophy 1 (OPA1), mutated in dominant optic atrophy, controls the shape of mitochondrial cristae keeping their junction tight during apoptosis (1), when proapoptotic BCL-2 family members BID induces a dramatic remodelling of the IM in order to grant cytochrome c mobilization from the cristae and ensure competent caspase activation. Tightness of the cristae correlates with oligomerization of two forms of OPA1, a soluble, intermembrane space one, and second one integral in the inner membrane. The cristae are also the site of oxidative phosphorylation, where the respiratory chain complexes are organized into supercomplexes in order to improve the efficiency of electron channelling (2). While the role of cristae remodelling in the amplification of the apoptotic cascade has been established, its consequences on mitochondrial function are unknown. We therefore decided to investigate if apoptotic cristae remodelling affects the activity and the structure of the mitochondrial respiratory chain supercomplexes (RCS), preferentially located in the cristae. BID impairs the activity and the structure of RCS, impacting on complex I dependent mitochondrial respiration which requires RCS assembly. These changes were abrogated by a novel alpha-6 helix BID mutant that is unable to induce cristae remodelling. The molecular basis of the assembly and stabilization of RCS are still unknown. We reasoned that OPA1, whose oligomers are disrupted during cristae remodelling, could play a role in the assembly and stability of RCS. A genetic analysis showed that *Opa1*-/- mitochondria still have individual respiratory chain complexes, but they fail to properly assemble RCS, resulting in lower RCS levels. This in turn impairs the growth rate of *Opa1*-/- cells when they are forced to use mitochondrial OXPHOS activity. Along the same line, expression of wt, but not of the cristae remodelling incompetent BID mutant in cells lacking Bak and Bax results in a reduced growth rate when energy production requires mitochondria. In conclusion, our data indicate that the shape of the cristae is essential for the assembly of the RCS and that during apoptosis the remodelling of the cristae affects mitochondrial respiratory efficiency.

STOP GOING IN CYCLES…USE MACS TECHNOLOGY FOR MITOCHONDRIA ISOLATION

Miltenyi-Biotec

Mitochondria play a central role in many cellular functions, including bioenergetics, apoptosis, and the lipid, iron, nucleotide, and aminoacid metabolism. There is strong evidence showing that mitochondria are implicated as key participants in neurological disorders, aging, myopathy, cancer and many other diseases. Since experimental conditions in isolated mitochondria can be controlled more precisely rather than in intact cells or tissues, isolated organelles provide a unique tool to investigate not only apoptosis, reactive oxygen species (ROS)2 production, and biogenetics but also mitochondrial DNA (mtDNA), mitochondrial RNA (mtRNA), and mitochondrial protein synthesis. For this reason mitochondria have become the subject of intense study in numerous fields, including biomedical research, drug discovery, and proteomics.
Isolation of mitochondria by current methods relies mainly on their chemical and physical properties. Here we describe an alternative approach to obtain functional mitochondria from human cells in a fast, reproducible, and standardized procedure.
The Mitochondria Isolation Kit developed by Miltenyi Biotec allows isolation of intact, viable mitochondria from human cells or tissues in less then 2 hours.
After cell lysis, the Translocase of the Outer mitochondrial Membrane 22 (TOM22) is targeted by anti-TOM22 MicroBeads in order to magnetically isolate functional human mitochondria.
Organelles isolated with MACS® Technology maintain their integrity, resulting in reliable and reproducible data in functional and biochemical assays; moreover immunomagnetic separation allows for the isolation of mitochondria independently of their size.
Higher yields (1) of intact, viable mitochondria are achieved by MACS® Technology, when compared to standard approaches, such as differential centrifugation or density gradient centrifugation.

(1) Hue-Tran Hornig-Do, Gritt Günther, Maria Bust , Patricia Lehnartz , Andreas Bosio,
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RNS AND ROS BIOLOGY

REDOX PROPERTIES OF NOVEL MITOCHONDRION-TARGETED QUERCETIN AND RESVERATROL DERIVATIVES

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"Mitochondriotropic" redox-active molecules provide a tool to intervene on a major cellular source of radical oxygen species (ROS). Mitochondrial ROS are involved in processes ranging from aging to ischemia/reperfusion damage, and have recently been shown to be a major determinant of the metastatic potential of cancers. Both oxidation-quenching and -enhancing activities would be useful in pathophysiological situations.

Since plant polyphenols are natural compounds which exhibit both antioxidant and pro-oxidant activity, depending on circumstances, we are developing polyphenol-based mitochondria-targeted molecules capable of acting as radical-scavengers or death-inducing pro-oxidants.

We have produced mitochondria-targeted derivatives of two model polyphenols, quercetin and resveratrol, by joining them to triphenylphosphonium, a membrane-permeant cation.

If these compounds are to display the desired redox activity in vivo, the chemical modifications introduced should not significantly alter the oxidation potential and the reactivity vs radical species of the parent compounds. To have a choice, we synthesised two different mitochondriotropic isomers of both quercetin and resveratrol: 3- or 7- (4-O-triphenylphosphoniumbutyl)-quercetin (3-QBTPI, 7-QBTPI), and 3- or 4'-(4-O-triphenylphosphoniumbutyl)-resveratrol (3-RBTPI, 4'-RBTPI). The redox properties of these derivatives were characterized through cyclic voltammetry and the DPPH reactivity assay, one of the most widely used methods to rank molecules on the basis of their ROS scavenging power; results were then compared to the properties of the parent polyphenols.

The two isomeric quercetin derivatives turned out to behave differently, with the 7-derivative having an oxidation potential very close to that of quercetin itself, and the 3-isomer a higher one. This difference between 3- and 7- isomers may be ascribed to the different stabilities of oxidation products. 7-QBTPI was also a more effective quencher of DPPH than 3-QBTPI. Thus the former isomer may be more suitable for our pourposes.

Predictably, resveratrol and its derivatives were oxidised at higher potentials than quercetin. The DPPH assay showed no significant difference between resveratrol and 4'-RBTPI, but it was not suitable to evaluate the radical scavenging properties of 3-RBTPI, since experimental conditions lead to its dimerization.

To verify whether a correlation exists between redox behaviour and biological action, evaluation of cytotoxicity/cytostaticity, mechanism of cell death, radical production and mitochondrial depolarization are under investigation on cultured cells with all the derivatives.

IN VITRO TOXICOLOGY OF MITOCHONDRIOTROPIC POLYPHENOLS

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Polyphenols display, at least in vitro, properties and effects of relevance for physiopathological conditions ranging from aging to cancer. Practical applications for these potentially useful compounds are made difficult by their low bioavailability. A priori, one way to circumvent this problem may be to cause their accumulation at the sites of action. Our group has synthesized a few derivatives of quercetin and resveratrol capable of accumulating into mitochondria. This property is conferred by the triphenylphosphonium group (TPP^+), a lipophylic cation which can diffuse through biomembranes and accumulate in regions held at negative electrical potential, such as the mitochondrial matrix and the cytoplasm. After verifying their mitochondriotropic behaviour in vitro and evaluating redox and radical scavenging properties, we investigated their effects on cultured cells. The compounds (μM range) proved cytotoxic, much more so for tumoural and fast-growing non-tumoural cells than for slow-growing ones. Mitochondriotropic quercetin derivatives induced a marked increase of ROS production, and all TPP-linked polyphenolic compounds caused cyclosporin A-insensitive mitochondrial depolarisation. We are investigating the mechanism(s) of death induction. The data indicate that these compounds are possible chemotherapeutic agents, and *in vivo* assays are planned to obtain a more realistic assessment of their potential.

FLAVOHEMOGLOBIN AND NO-DETOXIFICATION IN THE HUMAN PROTOZOAN PARASITE GIARDIA INTESTINALIS

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Flavohemoglobins (flavoHbs) are commonly found in bacteria and fungi, where they are considered to play a pivotal role in NO-detoxification by catalyzing the aerobic degradation of nitric oxide (NO) to nitrate [1]. *Giardia intestinalis*, a microaerophilic parasite causing one of the most common intestinal human infectious diseases worldwide [2], is the only pathogenic protozoon as yet identified coding for a flavoHb, based on genomic analysis [3]. In the present study, the recombinant *Giardia* flavoHb has been purified and functionally characterized by NO-amperometry and absorption spectroscopy, thus representing the first protozoan flavoHb to be investigated in detail [4]. The recombinant protein, as purified, contained 1 heme and ~ 0.3 FAD and displayed characteristic UV/visible absorption spectra in different redox and ligation states. In the presence of NADH, purified *Giardia* flavoHb metabolizes NO with high efficacy under aerobic conditions (TN = 116 ± 10 s^{-1} at 1 μM NO, T = 37 °C). The activity is $[O_2]$-dependent and characterized by an apparent K_M,O_2 = 22 ± 7 μM. As show by immunoblotting analysis, the protein is expressed at low levels in the trophozoites of *Giardia*, the vegetative cells of the parasite; accordingly, these cells aerobically metabolize NO with low efficacy. Interestingly, in response to nitrosative stress (24-hours incubation with = 5 mM nitrite) flavoHb expression is enhanced and the trophozoites thereby become able to metabolize NO efficiently, the activity being sensitive to both cyanide and carbon monoxide. The NO-donors S-nitrosoglutathione (GSNO) and DETA-NONOate mimicked the effect of nitrite on flavoHb expression. We propose that physiologically flavoHb contributes to NO detoxification in *G. intestinalis*.

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HCV INFECTION CAUSES ENHANCED MITOCHONDRIAL Ca^{2+}-SENSITIVE LIPID ACCUMULATION UP-REGULATING THE ACETYL CoA CARBOXYLASE ACTIVITY

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A hallmark of HCV-mediated diseases is altered lipid metabolism leading ultimately to liver steatosis in affected patients. In addition, a growing number of evidences proved that HCV protein expression causes unbalance of the intracellular redox state which can be brought back to mitochondrial-related dysfunctions.
In the present study we investigated the possible involvement of the HCV-related mitochondrial dysfunction in the steatogenetic process.
Cell lines: U2-OS cells inducibly expressing the full lenght HCV genome or subgenomic constructs coding for either structural or non structural HCV proteins under the control of a tetracycline-regulated gene expression system; Huh-7.5 human hepatome-derived cells transiently transfected by micro-electroporation with a full length HCV genome harboring a GFP insertion in the C-terminus of NS5A.
Irrespective of the in vitro cell system used, HCV protein expression resulted in upregulation of the Acetyl CoA carboxylase (ACC) both at the mRNA transcript and protein level. This was observed both in full length and in subgenomic HCV expressing cells. Moreover, the fractional inactive phosphorylated form of ACC was markedly decreased in HCV protein expressing cells. Consistently, an increased expression and phosphorylation of both Akt and GSK3β was observed. An enhaced intracellular lipid accumulation was directly detected by probe-assisted fluorescence confocal microscopy in HCV expressing cells as lipid droplets. Intriguingly ruthenium red (RR) and dantrolene treatment prevented completely the HCV-mediated increased lipid droplets formation.
This study indicates that HCV infection induces promotion of liponeogenesis mainly through upregulation of ACC, which is recognized as the key regulator of the fatty acid biosynthesis, both at the transcriptional/translactional and post-translational level. As ACC is known to be phosphorylated and inactivated by the GSK3β the activation of ACC is consistent with the observed increase of the phosphorylation states of Akt and GSK3β. These HCV-mediated changes might contribute to enhance liponeogenesis, as shown by the observed increase in lipid droplets content. Prevention of lipid accumulation in HCV expressing cells by the calcium channels inhibitors RR and dantrolene suggests a link to mitochondrial dysfunction. We speculate that the increased intramitochondrial calcium load and the consequent impairment of the OXPHOS system might possibly slow down the TCA cycle thereby resulting in accumulation of acetyl CoA and in its ACC-mediated channelling through the fatty acid synthesis. A ROS-mediated induction of phospho-Akt might further contribute to activation of ACC in HCV-infected cells.

MOLECULAR STRUCTURE AND FUNCTION OF MEMBRANE PROTEINS

STRUCTURE AND EVOLUTION OF MITOCHONDRIAL OUTER MEMBRANE PROTEINS

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Gram-negative bacteria are the ancestors of mitochondrial organelles. Consequently, both entities contain two surrounding lipid bilayers known as the inner and outer membranes. While protein synthesis in bacteria is accomplished in the cytoplasm, mitochondria import 90-99% of their protein ensemble from the cytosol in the opposite direction. In mitochondria four protein families including Sam50, VDAC, Tom40 and Mdm10 compose the set of integral β-barrel proteins embedded in the mitochondrial outer membrane (MOM). The 16-stranded Sam50 protein forms part of the sorting and assembly machinery (SAM) and shows a clear evolutionary relationship to members of the bacterial Omp85 family. By contrast, the evolution of VDAC and Tom40, both adopting the same fold cannot be traced to any bacterial precursor. This finding is in agreement with the specific function of Tom40 which was not existent in the enslaved bacterial precursor cell. Models of Tom40 and Sam50 have been developed using X-ray structures of related proteins. These models are analyzed with respect to conservation and charge properties yielding features related to their individual functions.

IS THE N-TERMINAL SEQUENCE IMPORTANT FOR THE FUNCTIONALITY OF THE VOLTAGE DEPENDENT ANION CHANNEL (VDAC)?

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In this paper we will review the results obtained in ours and in other's laboratories and focused on the structure and function of the N-terminal sequence of the Voltage-Dependent Anion Channels (VDAC). VDACs are the most abundant proteins of the mitochondrial outer membrane that form hydrophilic pore structures and are the main transport pathway for the exchange of metabolites into and from the mitochondria (1).
The structure of mammalian VDAC1 has been recently solved by NMR and crystallization experiments (2). It shows a rather compact transmembrane channel, formed by 19 amphipathic beta strands connected by short turns and loops, with the striking addition of the N-terminal moiety structured with α-helix segments. The N-terminal sequence is located inside the channel, forming a partial obstruction of the wide pore. CD and NMR spectroscopy showed that the 20 aa N-terminal peptide needs a special environment to form an ordered α-helix secondary structure. The conditions carrying the peptide to assume *in vitro* the structured α-helix were the presence of negative charges together with a hydrophobic environment (3). In addition it was shown that the deletion of the N-terminal does not change the mitochondrial targeting of the protein (3).
Experiments of swapping of the VDAC isoforms N-terminal ends showed that the functionality of VDAC is heavily influenced by the exchange of such moieties. In particular the isoform VDAC3, usually considered the least active of the three isoforms, becomes fully active upon exchange of its natural N-terminus with the same sequence from VDAC1 (4). This result will be commented also taking into account the global composition of these proteins.

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ACKNOWLEDGEMENTS
The financial support of FIRB RBRN07BMCT and PRIN MIUR 2008SW44CS_004 to VDP is acknowledged.

INTRINSIC UNCOUPLING: ADP AND PI MODULATION IN THE WT AND e-TRUNCATED ATP SYNTHASES OF ESCHERICHIA COLI

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The H^+/ATP ratio in the catalysis of ATP synthase has generally been considered a fixed parameter. However, Melandri and coworkers have recently shown that, in the ATP synthase of the photosynthetic bacterium *Rb.capsulatus*, this ratio can significantly decrease during ATP hydrolysis when the concentration of either ADP or P_i is maintained at a low level (Turina et al., 2004). We have reproduced similar phenomena in the ATP synthase from *E.coli*, both in inverted membranes (D'Alessandro et al., 2008) and in the purified reconstituted enzyme (manuscript in preparation).

We have then focused on searching for a possible structural feature involved in the phenomenon of intrinsic uncoupling. The e-subunit of the ATP-synthase is known as an endogenous inhibitor of the hydrolysis activity of the complex and appears to undergo drastic conformational changes between a non-inhibitory form (down-state) and an inhibitory form (up-state). In addition, the results of Cipriano & Dunn (2006) indicated that the C-terminal domain of this subunit played an important role in the coupling mechanism of the pump. We investigated the modulation of pumping efficiency in a C-terminally truncated e mutant. We have compared the ATP hydrolysis and the proton pumping activity in *E.coli* inverted membranes carrying either the WT e subunit or the $e_{88\text{-stop}}$ truncated form. Interestingly in the truncated mutant the inhibition of hydrolysis by P_i was largely lost; however, pre-incubation of the mutated enzyme with ADP at very low concentrations (apparent Kd = 0.7nM) restored the hydrolysis inhibition by P_i, together with the modulation of intrinsic uncoupling by this ligand. This indicates that, contrary to WT, the truncated mutant had lost the ADP bound at this high-affinity site during membrane preparation, evidently as a consequence of a lower affinity for ADP of the mutant relative to WT. Therefore, one of the effects of the C-terminal domain of e appears to be to strongly influence the affinity of at least one of the binding sites for ADP. The lack of this domain does not appear to abolish the modulability of coupling efficiency found in the WT, but instead to influence the extent of this modulation though a different binding of ADP.

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THE ROLE OF BOUND WATER MOLECULES IN STABILIZING THE PRIMARY CHARGE SEPARATION OF PHOTOSYNTHETIC REACTION CENTERS

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Theoretical and experimental studies indicate a critical role of the hydration water in the dynamics and function of soluble proteins [1]. In bacterial photosynthetic reaction centers (RC) the recombination kinetics of the light-induced primary charge separated state $P^+Q_A^-$ between the primary electron donor (P^+) and quinone acceptor (Q_A^-) is a sensitive probe of the internal RC dynamics. Hindrance of the RC dynamics (at low temperature in water-glycerol systems [2] or at room temperature in dehydrated trehalose glasses [3]) prevents stabilization of $P^+Q_A^-$, resulting in strongly accelerated and distributed charge recombination kinetics.

We have studied these effects in room temperature films of RCs from *Rhodobacter sphaeroides*, in the presence of the detergent N,N-dimethyldodecylamine N-oxide (LDAO) or octyl glucoside (OG), as a function of the residual water content under controlled relative humidity (RH). The water content was evaluated by FTIR spectroscopy from the area of the combination band of water around 5155 cm^{-1} [3]. The adsorption isotherms at 297 K, well described by the Hailwood and Horrobin equation, indicate a significant involvement of the protein detergent belt in water sorption, and reveal at least two populations of water molecules which interact differently with the RC-detergent complex.

Dehydration of the RC films strongly affects the kinetics of $P^+Q_A^-$ recombination, which upon decreasing the content of residual water become progressively faster and strongly distributed over a continuous spectrum of rate constant. At RH≅10% the average rate constant, $\langle k \rangle$, and the width σ of the rate distribution measured at room temperature are comparable with those reported for the hydrated system at cryogenic temperature [2], showing that extensive dehydration dramatically inhibits the interconversion between conformational substates of the RC. In films equilibrated at RH values lower than 10% a second, strongly non-exponential kinetic phase is resolved in the hundreds of μs time-scale. This faster phase appears to be related to the removal of tightly bound water molecules, critically involved in the stabilization of the primary charge separated state under physiological conditions.

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THE DRIVING FORCE OF LIFE

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Oxygenic photosynthesis is driven by two Photosystems I and II. Despite its enormous complexity, the plant PSI is arguably the most efficient nano-photochemical machine in Nature. It emerged as a homodimeric structure containing several chlorophyll molecules over 3.5 billion years ago, and has perfected its photoelectric properties ever since. The recently determined structure of plant PSI, which is at the top of the evolutionary tree of this kind of complexes, provided the first relatively high-resolution structural model of the supercomplex containing a reaction center and a peripheral antenna complexes. The structure provides a glimpse at the fine architecture of nature's efficient nano-photochemical machine that was perfected during long evolutionary time. Potential utilization of PSI for photoelectric devices will be discussed.

MITOCHONDRIAL PHYSIOLOGY AND PATHOLOGY
I I

BIOCHEMICAL CHARACTERIZATION ON POLYPEPTIDES OF HUMAN MRP6 PROTEIN

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The human gene ABCC6 maps on chromosome 16p13 and encodes the MRP6 (Multidrug Resistance Protein 6) protein of 1503 aminoacid residues. Together with MRP1, MRP2, MRP3 and MRP7, MRP6 belongs to a subgroup characterized by an additional N-terminal transmembrane associate domain (TMD0) [1] as well as two transmembrane domains not much conserved, TMD1 and TMD2, that bind substrates, and two hydrophilic domains, NBD1 and NBD2 (nucleotide binding domains), able to bind and hydrolyze ATP. The MRP6 is poorly characterized: the specific physiological function and the natural substrate(s) transported are currently unknown.

Mutations of ABCC6 gene cause Pseudoxanthoma elasticum (PXE), an inherited disorder characterized by the calcification of elastic fibers in skin, arteries and retina [2]. The structural determination of a large membrane protein such as MRP6 might be a difficult task mainly because membrane proteins are present at low levels and they are difficult to purify. An alternative to the study of the whole protein could be to study the protein domain per domain, as long the domains can be isolated as folded stable active polypeptides.

In order to perform a biochemical characterization of MRP6 we have overespressed in E.coli the 1-102 region of the MRP6 (TMD0), with a function poorly defined, and the NBD1 and NBD2 domains that present most of the PXE correlated mutations [3].

Preliminary information about the secondary structure of these domains were obtained by circular dichroism spectroscopy that showed that all polypeptides are structured.

Moreover, fluorescence experiments demonstrate that both NBD1 and NBD2 bind the nucleotides ATP, ADP and AMP with different affinity.

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MODULATION OF THE MITOCHONDRIAL PERMEABILITY TRANSITION BY QUINONES – THE CASE OF IDEBENONE

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Quinones affect the mitochondrial permeability transition pore (PTP), a high-conductance inner membrane channel modulated by the proton electrochemical gradient and by many signaling molecules. Detailed studies have allowed to assign quinones to three functional classes, i.e. inhibitors (which desensitize the PTP to Ca^{2+} and P_i), inducers (which sensitize the PTP) and apparently inactive quinones that compete with both inhibitors and inducers [1-3]; yet a clear structure-function activity correlation has not emerged, and major questions remain on the mechanism(s) through which quinones affect the PTP, an issue that has obvious pathophysiological and therapeutic implications. Quinones are indeed essential for both mitochondrial electron transfer and production of reactive oxygen species, and are involved in a variety of cellular functions. We have further investigated the mechanisms through which quinones affect the PTP and characterized in some detail the effects of idebenone on mitochondrial bioenergetics and PTP modulation. We found that (i) idebenone activates the PTP both in isolated mitochondria and *in situ*, with an effect that can be inhibited by Cyclosporin A (CsA); (ii) DTT prevents the PTP-inducing effects of idebenone, and promotes electron transfer from idebenone to complex III of the respiratory chain bypassing the lack of complex I activity; (iii) in the presence of DTT, idebenone considerably increases antimycin A-sensitive respiration both in normal and in XTC.UC1 thyroid oncocytoma cells bearing a disruptive frameshift mutation in the *MT-ND1* gene which impairs complex I assembly [4].

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MOLECULAR INVESTIGATION OF RIBOFLAVIN-RESPONSIVE MULTIPLE ACYL-CoA DEHYDROGENASE DEFICIENCY (RR-MAD) PATIENTS

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Riboflavin-responsive multiple acyl-CoA dehydrogenase deficiency (RR-MAD) is a lipid storage myopathy characterised by muscle weakness, decrease in fatty acid (FA) beta-oxidation capacity, low muscle carnitine, altered profiles of urinary organic acids and of plasma acyl-carnitine. We studied a relative large cohort of RR-MAD patients, performing a battery of biochemical and molecular tests to better characterise the metabolic disturbance of this disease and to explore the pathophysiological events (1-3). In particular we observed biochemical alterations of pre-therapy patient muscle samples included reduction in beta-oxidation and reduced activity of several flavin-dependent enzymes, such as short and medium-chain acyl coenzyme A dehydrogenases and complexes I and II of the respiratory chain. In the pre-treatment samples, flavin mononucleotide (FMN) and flavin dinucleotide (FAD) concentrations were also reduced. In some patients, an increased activity of mitochondrial FAD pyrophosphatase was observed (150 -273% of the mean value of controls) (1, 3), thus suggesting an altered regulation of mitochondrial flavin cofactor homeostasis (4). Proteomic investigation of muscle mitochondria in one RR-MAD patient (3) revealed decrease or absence of several proteins. All these deficiencies were completely rescued after riboflavin treatment. Proteomic data indicate that the enzymatic defects are multiple, coordinated, riboflavin-responsive and more extensive than previously described.
In patients with RR-MAD the molecular defect is still unknown. A disorder of mitochondrial flavin metabolism or transport has been proposed, for the several reasons, such as low intramitochondrial concentration of FMN and FAD, an increased activity of mitochondrial FAD pyrophosphatase and decreased activities and immunoreactive protein of several mitochondrial enzymes. However recently it was described that RR-MAD is associated with defects in electrontransfer flavoprotein dehydrogenase (ETFDH) gene in a large proportion of cases. Therefore we performed molecular investigation by sequencing the *ETFDH* gene to identify the genetic defects in this group of RR_MAD patients. The genomic sequence of each exon of *ETFDH,* including intron/exon boundaries has been PCR-amplified for 30 cycles. The PCR products have been purified and directly sequenced.

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MITOCHONDRIAL BIOGENESIS INDUCED BY PPARs AGONISTS IMPROVES VIABILITY OF LEBER'S HEREDITARY OPTIC NEUROPATHY (LHON) CYBRIDS

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LHON is a blinding disorder characterized by a rapid loss of central vision and optic atrophy, due to the selective degeneration of retinal ganglion cells (RGCs). This maternally inherited disease is caused by mutations in mitochondrial DNA (mtDNA) encoded genes of respiratory chain Complex I. The most common mutations are 11778/ND4, 14484/ND6 and 3460/ND1, found in 90% of LHON cases. In some mitochondrial diseases, a cellular strategy to supply to the energetic defect due to mtDNA mutations is the induction of mitochondrial proliferation. Our previous studies demonstrated that this compensatory mechanism is also evident in LHON patients: mtDNA levels resulted higher in LHON patients, compared to controls, and this increase was more consistent in carriers (individuals harboring LHON mutations but not clinically affected). Thus, those subjects that are able to induce a more efficient mitochondrial biogenesis resulted less prone to develop the disease.
Mitochondrial biogenesis is a complex process, finely tuned by specific transcription factors and coactivators, involving the coordinated expression of mitochondrial and nuclear genes. Recently, it has been shown that treatment with bezafibrate (BF), a commercially available drug, can improve mitochondrial function in a myopathy mouse model and in cells with moderate respiratory chain defects, via mitochondrial biogenesis activation.
In this preliminary study we tested the effect of two PPARs agonists, bezafibrate and rosiglitazone (RGZ), on LHON cybrids, attempting to stimulate mitochondrial biogenesis and rescue their defective phenotype. We found, with both drugs, a time-dependent increase of mitochondrial proteins in control cell lines, with a maximum after 96 hours. Thus, we analyzed the cellular response to these drugs evaluating mtDNA copy number and mitochondrial protein levels at this time point; we found that both drugs induced an increase of mtDNA copy number and mitochondrial proteins, but RGZ resulted more efficient in the stimulation of mtDNA replication. Moreover, the treatment with BF and RGZ resulted able to rescue 11778/ND4 cybrids from galactose-induced cell death, with a significant improvement of cell viability after 48 and 72 hours.
This preliminary study suggests that PPARs agonists stimulate mitochondrial biogenesis and can improve the mitochondrial function in LHON cybrids, highlighting how the mild LHON mutations may be easily compensated. Based on the consolidation of these results PPARs agonists may be considered as potential treatment in clinical trials enrolling LHON patients. A detailed dissection of the mitochondrial biogenesis pathways at work in unaffected LHON carriers compared to the affected individuals may further shed light on other therapeutic strategies.

TOWARD A MITOCHONDRIAL THERAPY FOR COLLAGEN VI MUSCULAR DYSTROPHIES

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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 in skeletal muscle. We demonstrated that these dysfunctions are caused by inappropriate opening of the permeability transition pore (PTP), a mitochondrial inner membrane channel that plays a role in several forms of cell death. 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. These encouraging results have tremendous implications for the therapeutic perspectives of ColVI muscular dystrophies, but long-term treatment with CsA exposes the patients to the untoward effects of immunosuppression, which may favour life-threatening infections. We have therefore evaluated the effects of treatment with the Cs derivative Debio 025 (D-MethylAlanine^{3}-EthylValine^{4}-cyclosporin) on *Col6a1*$^{-/-}$ mice. Debio 025 maintains the ability to bind to, and inhibit, CyPs but does not inhibit calcineurin, the target of CsA involved in the immunosuppressive effect. 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 that treatment with Debio 025: (i) does not inhibit calcineurin, yet it desensitizes the PTP *in vivo*; (ii) prevents mitochondrial dysfunction; and (iii) normalizes 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 Ca^{2+}-REGULATED MITOCHONDRIAL (PERMEABILITY TRANSITION) PORE IN DROSOPHILA MELANOGASTER

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The mitochondrial permeability transition (PT) describes a process of Ca^{2+}-dependent, tightly regulated increase in the permeability of the inner mitochondrial membrane that has been discovered and studied mostly in mammals. The PT, which is critically involved in cell death, is due to the opening of the mitochondrial permeability transition pore (PTP), a high-conductance inner membrane channel with unknown structure. Although data are available also in yeast and plants, it is not clear whether the permeability changes observed in these organisms can be ascribed to the same molecular events involved in the mPT of mammals. We have studied the properties of the PT in mitochondria from the fruit fly *Drosophila melanogaster*. Experiments were carried out in permeabilized *Drosophila* embryonic S_2R^+ cells, where we demonstrate the occurrence of ruthenium red-sensitive Ca^{2+} uptake as well as of a ruthenium red-insensitive Ca^{2+} release following matrix Ca^{2+} overload (which in mammals is caused by opening of the PTP). Ca^{2+} release was insensitive to CsA, Ub0 and ADP, well-known inhibitors of the mammalian PTP; but was inhibited by Mg^{2+} (as is the PTP of all species) and Pi (as is the "pore" of yeast). Ca^{2+}-induced Ca^{2+} release could be triggered by the thiol reactive compound N-ethylmaleimide and by the dithiol oxidant diamide, indicating the existence of regulatory redox-sensitive sites, as is known for the mammalian PTP. Our results suggest that *Drosophila* mitochondria may possess a Ca^{2+}-regulated permeability pathway with features intermediate between the "pore" of yeast and the PTP of mammals.