GIBB 2006

Comitato scientifico

Direttivo del Gruppo Italiano
di Biomembrane e Bioenergetica

Comitato Organizzatore

Anna Maria Ghelli, Anna Maria Porcelli,
Claudia Zanna, Michela Rugolo

Dipartimento di Biologia Evoluzionistica Sperimentale
Università degli studi di Bologna

Con il patrocinio dell'
Università degli Studi di Bologna

PROGRAM

AND

ABSTRACT BOOK

THURSDAY, 15TH JUNE 2006

13.00 – 15.00 Registration

15.00 Opening addresses

MOLECULAR STRUCTURES OF MEMBRANE PROTEIN COMPLEXES

Chairman: Sergio Papa (*University of Bari)*

15.10 Structural complexity of neuronal nicotinic receptors.
Francesco Clementi *(University of Milano)*

15.50 Oligomeric states of F_0F_1ATP-synthase in bovine heart mitochondria.
E. Bisetto, F. Di Pancrazio, V. Alverdi, I. Mavelli, G. Lippe *(University of Udine)*

16.10 Oligomeric properties of the ATP synthase inhibitory peptide IF1 from *Saccharomyces cerevisiae.*
E. Bisetto, V. Corves, C. Sigalat, G. Lippe, F. Haraux *(University of Udine)*

16.30 Effect of ADP and Pi on the intrinsic uncoupling in the ATP synthase of *E.coli*.
M. D'Alessandro, P. Turina and B. A. Melandri *(University of Bologna)*

16.50 Can the proton pumping efficiency of ATP synthase be regulated?
A. Rebecchi, P. Turina, B. A. Melandri (*University of Bologna)*

17.10 – 17.30 COFFEE BREAK

Chairman: Paolo Sarti (*University of Rome)*

17.30 Stabilization of the charge separated state and distribution of the ubiquinone pool in reaction center-light harvesting complexes purified from *Rhodobacter shaeroides*.
M. Dezi, F. Francia, G. Palazzo, A. Maliardi, G. Venturoli (*University of Bologna)*

17.50 X-ray absorption studies of Zn^{2+} binding sites in bacterial, avian and bovine cytochrome bc_1 complexes.
L. Giachini, F. Francia, D-W. Lee, F. Daldal, L-S. Huang, E.A. Berry, T. Cocco, S. Papa, F. Boscherini , G. Venturoli (*University of Bologna)*

18.10 The Cu_b-lacking cytochrome *BD* oxidase and nitric oxide.
E. Forte, V.B. Borisov, A. Giuffrè, M. Brunori, F. M. Scandurra, A.A. Konstantinov, P. Sarti (*University of Rome "La Sapienza")*

18.30 The flux control of cytochrome c oxidase on cell respiration is controlled by mitochondrial $\Delta\mu H^+$ and mediated by cardiolipin.

***G. Quarato*, C. Piccoli, R. Scrima, D. Boffoli, N. Capitanio** *(University of Foggia)*

20.30 DINNER

FRIDAY, 16th JUNE 2006

NITRIC OXIDE AND ROS BIOLOGY

Chairman: Giorgio Lenaz (*University of Bologna)*

9.00 Role of nitric oxide in mitochondrial function and biogenesis.
Emilio Clementi *(Univ. di Milano)*

9.40 The role of flavodiiron proteins in nitric oxide detoxification.
J. B. Vicente, M. C. Justino, L.M. Saraiva, M.Teixeira, F. M. Scandurra, E.Forte, M. Brunori , P. Sarti, A. Giuffrè (*University of Rome "La Sapienza")*

10.00 Correlative analysis between hematopoietic stem cells differentiation and mitochondriogenesis.
A. D'Aprile, C. Piccoli, R. Scrima, L. Lecce, D. Boffoli, A. Tabilio, N. Capitanio *(University of Foggia)*

10.20 Melatonin protects against mitochondrial dysfunction associated with cardiac ischemia-reperfusion by preventing ROS-induced cardiolipin oxidation.
G. Petrosillo, N. Di Venosa, M. Pistolese, G. Casanova, E. Tiravanti, G. Colantuono, A. Federici, F.M. Ruggiero, G. Paradies (*University of Bari)*

10.40 – 11.10 COFFEE BREAK

Chairman: Giuseppe Paradies (*University of Bari)*

11.10 Exploiting the effect of complex I inhibitors and the role of endogenous/ exogenous quinones on ROS production and on NADH-DCIP reductase activity.
R. Fato, C. Bergamini, S. Leoni, G. Lenaz (*University of Bologna)*

11.30 Succinate control of H_20_2 generation in mitochondria.
F. Zoccarato, L. Cavallini, S. Bortolami, E. Comellato and A. Alexandre (*University of Padova)*

11.50 Effects of inhibitors of NAD(P)H oxidase on glucose transport activity in a leukaemic cell line.
C. Prata, T. Maraldi, D. Fiorentini, L. Zambonin, G. Hakim, L. Landi *(University of Bologna)*

12.10 Mitochondrial oxidative metabolism in interleukin 7-engineered stromal cells.
R. Scrima, C. Piccoli, M. Ripoli, A. D'Aprile, D. Boffoli, A. Tabilio, S. Papa,

N. Capitanio *(University of Foggia)*

12.30 **The differentiation process of human monocyte-derived dendritic cells: possible role of mitochondria and reactive oxygen species.**
M.Di Paola, C.Oliveros Celis, A.Del Prete, P.Zaccagnino, G.Santoro, M.Lorusso *(University of Bari)*

13:00 **LUNCH**

MITOCHONDRIAL DYSFUNCTIONS

Chairman: Luca Scorrano *(Dulbecco-Telethon Institute, VIMM, Padova)*

15.00 **Paraplegin-AFG3L2 complex, a large mitochondrial metalloprotease with a role in neurodegeneration and not only**
Giorgio Casari *(Università Vita-Salute, Milano)*

15.40 **Generation of a transgenic mouse model overexpressing OPA1, a mitochondria-shaping protein mutated in dominant optic atrophy.**
V. Costa, S. Cipolat, L. Scorrano (*Dulbecco-Telethon Institute, VIMM, Padova)*

16.00 **OPA1 controls apoptotic cristae remodelling independently from mitochondrial fusion.**
C.Frezza, S. Cipolat, O. Martins de Brito, M. Micaroni, G.V. Beznoussenko, T. Rudka, D. Bartoli, R.S. Polishuck, N.N. Danial, B. De Strooper, L.Scorrano *(Dulbecco-Telethon Institute, VIMM, Padova)*

16.20 **Mitochondrial rhomboid PARL regulates cytochrome c release during apoptosis via OPA1-dependent cristae remodeling.**
S. Cipolat, T. Rudka, D. Hartmann, V. Costa, L. Serneels, K. Craessaerts, K. Metzger, C. Frezza, W. Annaert, L. D'Adamio, C. Derks, T.Dejaegere, L. Pellegrini, R. D'Hooge , L. Scorrano' B. De Strooper *(Dulbecco-Telethon Institute, VIMM, Padova)*

16.40 **Energetic efficiency and mitochondrial fusion in fibroblasts from Dominant Optic Atrophy patients bearing the C.2708delTTAG OPA1 mutation.**
C. Zanna, A. Ghelli, A. M. Porcelli, M. Karbowski, R.J. Youle, V.Carelli, M.Rugolo (*University of Bologna)*

17.00 – 17.20 COFFEE BREAK

Chairman: Giancarlo Solaini (*University of Bologna)*

17.20 **Functional and molecular responses to stress conditions in heteroplasmic MELAS 3243 RD cybrids.**
L. Vergani, E. Loro, A. Malena, S. Cazzorla, A. Marchetto *(University of Padova)*

17.40 **Comparative bioenergetic studies of cells carrying the 8993T>G or 8993T>C mitochondrial DNA mutations.**

***G. Sgarbi**, A. Baracca, M.Mattiazzi, V. Carelli, G. Lenaz, G.Solaini* (*University of Bologna)*

18.00 **New insights in the pathophysiology of riboflavin-responsive multiple acyl-CoA dehydrogenase deficiency (RR-MAD).**
C.Brizio, E. Gianazza, A. Russell, R. Wait, C. Angelini, F. Dabbeni-Sala, D. Bufano, I. Eberini, B. Lenger, M. Barile, L. Vergani *(University of Padova*

18.20 **Megacarioblastic leukemia and mitochondria: a case report.**
***M. Ripoli**, C. Piccoli, R. Scrima, O. Cela, D. Boffoli, A. Tabilio, M. Carella, N. Capitanio* (*University of Foggia)*

19,00 **MEETING FOR GIBB MEMBERS**

20,30 **SOCIAL DINNER**

SATURDAY, 17th JUNE 2006

MEMBRANES AND TRANSPORT

Chairman: Cesare Indiveri (*University of Arcavacata di Rende)*

9.00 **Regulated exo-endocytosis, an expanding world: properties and role of a new exocytic organelle, the enlargeosome.**
Jacopo Meldolesi *(Università Vita-Salute, Milano)*

9.40 **Functional and structural characterization of the N-terminal peptide of the mitochondrial VDAC1.**
***V. De Pinto**, A. Messina, R. Aiello, F. Tomasello, D. La Mendola, A., Magrì, D. Milardi, G. Pappalardo* *(University of Catania)*

10.00 **Dynamic aspects of the mitochondrial carnitine/acylcarnitine carrier structure, as revealed by chemical modification of four replacement cys mutants.**
***C. Indiveri**, A.Tonazzi, N. Giangregorio, F. Palmieri* *(University of Arcavacata di Rende)*

10.20 **Biotin-tagging of bilitranslocase in the intact liver as a mean for its detection and purification by avidin-biotin techniques.**
***R. Cecotti**, S. Passamonti* *(University of Trieste)*

10.40 **Expression and function of bilitranslocase in vascular endothelial cells.**
***M. Terdoslavich**, A.Maestro, A. Kuku, V. Nicolin, G. Decorti, S. Passamonti* *(University of Trieste)*

11.00- 11.20 COFFEE BREAK

INTEGRATED CELLULAR FUNCTIONS

Chairman: Nazzareno Capitanio (*University of Foggia)*

11.20 **cAMP-dependent protein kinase and A-kinase anchor proteins in the inner compartment of mammalian heart mitochondria**
A. Signorile, A.M. Sardanelli, R. Nuzzi, D.De Rasmo, Z. Technikova-Dobrova, Z. Drahota, A.Pica, A. Occhiello, S. Papa

11.40 **The energetic failure caused by severe mutations in the mitochondrial genome is not rescued by Bcl-2 overexpression**
M. Hoque, A.M. Porcelli, A. Ghelli, L. Iommarini, M. Rugolo (*University of Bologna)*

12.00 **NASH induces expression of UCP2 and mitochondria respiratory impairment in liver hepatocytes**
G. Serviddio, R.Tamborra, F.Bellanti, T.Rollo, A. Romano, E.Altomare, G.Vendemiale (*University of Foggia)*

12.20 **Role of calcium in mitochondrial dysfunctions induced by hepatitis C virus infection.**
C. Piccoli, R. Scrima, A. D'Aprile, G. Quarato, M. Ripoli, L. Lecce, D. Boffoli, D. Moradpour, N. Capitanio (*University of Foggia)*

12.40 **Species-specific stimulation of the mitochondrial permeability transition by norbormide analogues.**
A. Zulian, V. Petronilli, P. Bernardi, S. Bova, F. Dabbeni-Sala, G. Cargnelli, M. Cavalli, F. Ricchelli (*University of Padova)*

12.50 **Closure of the meeting**

ABSTRACTS

INVITED LECTURE

STRUCTURAL COMPLEXITY OF NEURONAL NICOTINIC RECEPTORS.

Francesco Clementi

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Via Vanvitelli 32, 20129 Milano, Italy.

OLIGOMERIC STATES OF F0F1ATP-SYNTHASE IN BOVINE HEART MITOCHONDRIA

Elena Bisetto, Francesca Di Pancrazio, Vera Alverdi, Irene Mavelli, Giovanna Lippe

Dipartimento di Scienze e Tecnologie Biomediche e M.A.T.I. Centro di Eccellenza, Università di Udine.

The demonstration of ATPsynthase complexes associated to form dimers and oligomers (1,2,3) and even supracomplexes with other proteins (4) in the inner mitochondrial membrane, as well as of their presence in plasma membrane (5) suggests a new dynamic view of ATPsynthase. The structural properties of the oligomeric forms are much better characterized in yeast with respect to mammals, in spite of the recent electron microscopy images of the dimers in bovine heart mitochondria (6). In addition, the involvement of ATPsynthase oligomerization in mitochondrial coupling and cristae biogenesis is well documented in yeast (3). In contrast, in mammals, the structural and functional roles of dimers/oligomers remain to be clarified. We investigated the susceptibility to in situ proteolysis of the oligomeric states of ATPsynthase in mitoplasts and in submitochondrial particles (SMP), which contain similar amounts of monomers/dimers and oligomers. Both membrane preparations were treated with chymotrypsin under controlled conditions which maintained dimers/oligomers, as assayed by 1D BN-PAGE of detergent extracts. In mitoplasts fragmentation of F1 subunits selectively in the monomer was observed, while in SMP a similar pattern of F1 degradation of all oligomeric forms occurred, as demonstrated by 2D SDS-PAGE, Western blot and mass spectrometry. The results obtained in SMP suggest that the accessibility to F1 subunits is similar in all oligomeric states, in accordance with the essential role of F0 subunits in dimers/oligomers stabilization (1,3,6). Similar F1 accessibility is also suggested by the finding that IF1 contents of monomers/dimers/oligomers was found to be similar in mitochondria, indicating that enzyme oligomerization does not strongly affect IF1 binding to F1 sector. The higher susceptibility to proteolysis of the monomers in mitoplasts, where the native morphology of inner membrane is maintained, may be consequence of their localization in different membrane-subcompartments. The idea that the different ATPsynthase states may play distinct functional roles in mitochondria (7) is in accordance with different sub-localisations within the membrane. Functions like control of enzymatic activity have been also assigned to ATPsynthase oligomerization (7). However, if this is the case, IF1 seems to be not involved.

1. Tomasetig L. et al., 2002 *Biochim Biophys Acta* 1556, 133-41
2. Di Pancrazio F. et al., 2005 *Proteomics* 6, 921-26
3. Bornhövd C et al., 2006 *JBC* in press
4. Ardehali H. et al., *Proc Natl Acad Sci* 101, 11880-85
5. Bae T.J et al 2004, Proteomics 4, 3536-48
6. Minnauro-Sanmiguel F. et al., 2005 *Proc Natl Acad Sci* 102,12356-8
7. Rexroth S. et al. 2004 *Biochim.Biophys.Acta* 1658, 202-11

OLIGOMERIC PROPERTIES OF THE ATP SYNTHASE INHIBITORY PEPTIDE IF1 FROM *SACCHAROMYCES CEREVISIAE*

Elena Bisetto[1,2], Vincent Corvest[1], Claude Sigalat[1], Giovanna Lippe[2], Francis Haraux[1]

[1] Service de Bioénergétique, Département de Biologie Joliot-Curie and CNRS-URA 2096, CEA Saclay, F 91191 Gif-sur-Yvette, France [2] Dipartimento di Scienze e Tecnologie Biomediche & M.A.T.I. Center of Excellence, University of Udine, p.le Kolbe 4, 33100 Udine, Italia

Mitochondrial ATP synthases are regulated by inhibitory peptides (IF1) that bind at a catalytic interface of F1 sector in 1:1 stoichiometry, inhibition being favoured by a decrease protonmotive force and by the presence of MgATP. In bovine species, the inhibitory form of IF1 is thought to be dimeric and favoured by low pH whereas high pH favours the formation of a non-inhibitory tetramer (1). bIF1 (*B. taurus*) is 84-residues long and yIF1 (*S. cerevisiae*) is 63 residues-long, the difference in size being mainly due to a shorter C-terminal part in yIF1. *S. cerevisiae* also contains a second inhibitory peptide homologous to yIF1 and called STF1, the role of which is unknown. yIF1 and STF1 are thought to exist under monomeric and dimeric forms, but the relationship between their oligomeric state and their inhibitory power is uncertain (2).

To know the relationship between the oligomeric state of yIF1 and its inhibitory power, we have engineered redox-dependent artificial dimers of this peptide by mutating into cysteins selected residues, the bovine homologous of which face together or not in the published structure of tetrameric bIF1 (3). $CuCl_2$-mediated crosslinking occurred in a few seconds regardless the position of the mutated residue(s). yIF1 dimers crosslinked in the C-terminal part could still rapidly inhibit ATPase activity of isolated F1-ATPase, submitochondrial particles (SMP) or alamethicin-treated mitochondria (4), whereas yIF1 dimers or oligomers crosslinked in the N-terminal were no more inhibitory. In the absence of $CuCl_2$, the degree of crosslinking was found dependent of the position of the mutation. Our data suggest that the natural interface of dimerization of yIF1 corresponds to the inter-dimers interface of tetrameric bIF1. The functional study of the dimer crosslinked in the C-terminal part, that mimicks the natural bIF1 dimer, also proves that such a dimer does inhibit ATPase activity, even in the matricial environment.

1. Cabezón, E. et al., 2000 *J. Biol. Chem*. 275, 25460-25464
2. Cabezón, E. et al., 2002 *J. Biol. Chem*. 277, 41334-41341
3. Cabezón, E. et al., 2001 *EMBO J*. 20, 6990-6996
4. Venard, R. et al., 2003 *Biochemistry* 42, 7626-36

EFFECT OF ADP AND PI ON THE INTRINSIC UNCOUPLING IN THE ATP SYNTHASE OF *E.COLI*.

Manuela D'Alessandro, Paola Turina, B. Andrea Melandri

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A phenomenon of intrinsic uncoupling has recently been shown in the ATP synthase of the photosynthetic bacterium *Rhodobacter capsulatus* (1). A progressively lower level of ATP-induced steady state ΔpH was observed by decreasing ADP or Pi concentrations, in spite of unaffected or increasing ATP hydrolysis.

Given the possible mechanistic and physiological significance of this phenomenon, we have looked for its occurrence also in the ATP synthase of *E.coli*. The ATP hydrolysis activity and proton pumping of isolated native membranes from *E.coli*, isolated from both a superproducing and a not-superproducing strain, have been measured and compared as a function of ADP and Pi concentration.

The ATP hydrolysis activity was inhibited by Pi with a Kd of 80 µM. When the ADP concentration was progressively decreased by increasing amounts of an ADP trap, the Kd increased progressively up in the millimolar range. In addition, the relative extent of this inhibition decreased with decreasing ADP. In contrast to this monotonic inhibition, the steady-state proton pumping activity of the enzyme, as estimated under the same experimental conditions both by the fluorescence quenching of the ΔpH-sensitive probe ACMA, and by the $\Delta\varphi$ -sensitive probe oxonol, showed a clearly biphasic progression, increasing from 0 up to approximately 200 µM Pi, and decreasing at higher Pi concentrations.

The inhibition of ATP hydrolysis by ADP showed a Kd in the micromolar range, which was similarly increased by decreasing Pi concentrations. Similarly as to what seen in the case of Pi, the dependence of steady-state ΔpH on ADP concentration was biphasic.

These data indicate that a switch from a partially uncoupled state of ATP synthase to a coupled state is induced by the simultaneous presence of ADP and Pi.

1. Turina P., Giovannini D., Gubellini F. and Melandri B.A. (2004) *Biochemistry* 43: 11126-34.

CAN THE PROTON PUMPING EFFICIENCY OF ATP SYNTHASE BE REGULATED?

Alberto Rebecchi, Paola Turina, B. Andrea Melandri

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The ATP synthase in chromatophores of *Rhodobacter caspulatus* can generate a transmembrane pH difference coupled to the hydrolysis of ATP. Under experimental conditions in which the ADP concentration was low (i.e. small concentration of ATP-containing ADP as contaminant- and of membranes) the rate of hydrolysis was rather insensitive to the depletion of ADP in the assay medium by an ATP regenerating system (phosphoenolpyruvate (PEP) and pyruvate kinase (PK)). The steady state values of ΔpH were however drastically reduced as a consequence of ADP depletion. The clamped concentrations of ADP obtained using different PK activities in the assay medium could be calculated and an apparent $K_d \approx 0.5$ µM was estimated. The extent of proton uptake was also strongly dependent on the addition of phosphate (P_i) to the assay medium. The K_d for this effect was about 70 µM.
The data discussed above were obtained using ACMA, a fluorescent probe very sensitive to small ΔpH. The effects of ADP and Pi as regulators of proton pumping were subsequently confirmed quantitatively using 9-amino-acridine, a less sensitive probe whose response to ΔpH can be more easily calibrated. In this case, higher concentrations of ATP and of membranes were required, generating higher levels of ADP when no ADP trap was present. Differences in the initial rates of pumping and of steady state ΔpH were again recorded: when the ADP was decreased to submicromolar concentrations by high levels of PK, the steady state ΔpH was decreased from 3.7 to 2.8 units, while the hydrolysis rate not only did not decrease, but instead increased several fold, due to the significant removal of inhibitory ADP under these experimental conditions.
We interpret these data as indicating a drastic decrease of proton pumping efficiency, a phenomenon which can be attributed to an intrinsic coupling within the ATP synthase modulated by the substrates of ATP hydrolysis. More precisely, ADP and phosphate induce a functional state of the ATP synthase competent for a tightly coupled proton pumping, while the depletion of either one of these two ligands favours an inefficient (slipping) functional state. The switch between these states can probably be related to the structural change in the C-terminal α-helical hairpin of the ε-subunit, from the extended conformation, in which ATP hydrolysis is tightly coupled to proton pumping, to the retracted conformation, in which ATP hydrolysis and proton pumping are loosely coupled.

STABILIZATION OF THE CHARGE SEPARATED STATE AND DISTRIBUTION OF THE UBIQUINONE POOL IN REACTION CENTER – LIGHT HARVESTING COMPLEXES PURIFIED FROM *RHODOBACTER SPHAEROIDES*.

Manuela Dezi[*], Francesco Francia[*], Gerardo Palazzo[#], Antonia Mallardi[¶], Giovanni Venturoli[*, §]

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We have recently shown that in the reaction center – light harvesting complex 1 (RC-LH1) purified from the photosynthetic bacterium *Rhodobacter sphaeroides*, at pH=7.8, flash-induced $P^+Q_B^-$ recombines with an average rate constant ($\langle k \rangle \approx 0.3\ s^{-1}$), significantly smaller than that measured in RC deprived of the LH1 ($\langle k \rangle \approx 1\ s^{-1}$), indicating that the $P^+Q_B^-$ state is energetically stabilized in core complexes [1]. The pH dependence of the $P^+Q_B^-$ recombination kinetics has been examined in dimeric and monomeric forms of RC-LH1 and compared with that observed in RC deprived of the antenna. At 6.5 < pH < 8.5 recombination is essentially pH independent and significantly slower in all forms of core complexes as compared to LH1-deprived RCs. At increasing pH values, however, where the recombination rate increases, this stabilization effect decreases progressively and vanishes at pH > 10.5, indicating the involvement of protonatable groups. The recombination kinetics becomes progressively distributed at pH > 9. The width of the rate constant distribution ($\sigma \cong 0.3\ s^{-1}$ at pH < 9.0) increases by more than one order of magnitude at pH 11.0, suggesting a variety of conformations, possibly differing in the protonation state. The observed pH dependence of σ could be explained when assuming that such conformations interconvert at a rate which is comparable to the rate of charge recombination at physiological pH values but is considerably lower at high pH values. Under this condition the conformational heterogeneity becomes therefore observable. A similar behaviour was observed in chromatophores of *Rhodobacter capsulatus* FJ2, a c_2- and c_y-minus strain, in which the kinetics of $P^+Q_B^-$ recombination could be accurately studied by avoiding any interference due to exogenous electron donors/acceptors.

The lipid complement of the RC-LH1 complexes, determined by Inductively Coupled Plasma Emission Spectroscopy of phosphorous, ranges between 200 and 400 phospolipid molecules per RC. A large ubiquinone (UQ) pool, varying from 15 to 30 UQ molecules per RC was systematically found to be associated with the core complexes. When similar determinations are performed in chromatophores, it appears that the effective UQ concentration in the lipid phase of core complexes is at least three times higher than the average UQ concentration in the intact membrane. This finding argues strongly in favour of an *in vivo* heterogeneity in the distribution of the quinone pool within the chromatophore bilayer.

1. Francia,F., Dezi,M., Rebecchi,A., Mallardi,A., Palazzo,G. , Melandri,B.A., Venturoli,G. (2004) Biochemistry 43, 14199-14210

X-RAY ABSORPTION STUDIES OF ZN^{2+} BINDING SITES IN BACTERIAL, AVIAN AND BOVINE CYTOCHROME BC_1 COMPLEXES.

L. Giachini[*, ‡], F. Francia[#], D-W. Lee[¶], F. Daldal[¶], L-S. Huang[||], E.A. Berry[||], T. Cocco[§], S. Papa[§], F. Boscherini[*, ‡], G. Venturoli[#, ‡]

[*]Department of Physics, University of Bologna, Italy; [#]Department of Biology, University of Bologna, Italy; [¶]Department of Biology, University of Pensylvania, Philadelphia, PA, USA; [||]Laurence Berkeley National Laboratory, Berkeley, CA, USA; [§]Department of Medical Biochemistry, Biology and Physics, University of Bari, Italy; [‡]CNISM, Italy.

Zn^{2+} is a well established inhibitor of the bovine mitochondrial cytochrome (cyt) bc_1 complex (1). In bacterial cyt bc_1 complexes Zn^{2+} decelerates electron transfer and transmembrane voltage generation (2). It has been proposed that Zn^{2+} binds close to the Q_0 site of the complex, blocking the proton release channel(s). To determine the coordination geometry of the bound metal and locate the cluster of binding residues we performed Zn K-edge X-ray Absorption Spectroscopy (XAS) measurements on Zn incubated samples of avian, bovine and bacterial (from *Rhodobacter capsulatus*) cyt bc_1 complexes.

EXAFS data were analysed using ab-initio theoretical amplitude and phase shift functions. Combining first shell and multi shell-multiple scattering analysis we found that in the bacterial complex Zn is penta-coordinated. The analysis suggests three nitrogens (or two nitrogens and one oxygen) at $\cong$ 2.06 Å and two oxygen atoms at $\cong$ 2.20 Å. The best fit is obtained for a cluster formed by two histidine and one glutamic/aspartic acid bound in a bidentate arrangement. The other coordinated residue can be either a glutamic/aspartic acid bound in a monodentate arrangement or a glutamine. In both the avian and the bovine complexes zinc is tetra-coordinated, binding three nitrogens at $\cong$ 2.04 Å and one oxygen at $\cong$ 1.99 Å. The best fit is obtained for a cluster formed by two histidine, one glutamic/aspartic acid bound in a monodentate arrangement and a lysine.

X-ray diffraction measurements performed on avian cyt bc_1 complex show the presence of two Zn atoms (3). One of the sites, which might interfere with the egress of H^+, is consistent with the cluster identified by XAFS analysis, being located in the proximity of His21, Asp253, Glu255, His268 and Lys270. Structurally homologous sites can be identified in the bovine (His267, His121, Asp254, Lys269, Asn255) and in the bacterial complex (His291, His276, Asp278, Glu295, Gln153), which well fit the information obtained by XAFS. As a whole, this comparative analysis suggests a common structural basis for the mechanism of Zn^{2+} inhibition.

1. Lorusso, M., Cocco, T., Sardanelli, A.M., Minuto, M., Bonomi, S., Papa, S. (1991) Eur. J. Biochem. 197, 555-561
2. Klishin, S.S., Junge, W., Mulkidjanian, A.Y. (2002) Biochim. Biophys. Acta 1553, 177-182
3. Berry, E.A., Zhang, Z., Bellamy, H.D., Huang, L. (2000) Biochim. Biophys. Acta 1459, 440-448

THE CU_B-LACKING CYTOCHROME *BD* OXIDASE AND NITRIC OXIDE

Elena Forte[1], Vitaly B. Borisov[2], Alessandro Giuffrè[1], Maurizio Brunori[1], Francesca M. Scandurra[1], Alexander A. Konstantinov[2], Paolo Sarti[1]

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Cytochrome *bd* is a bacterial respiratory quinol oxidase, which unlike the haem-copper oxidases, does not contain Cu_B in the active site. The reduction of O_2 to H_2O occurs at the heme *d* in the active site; the reaction is electrogenic, but not coupled to proton pumping (1). It has been suggested that cytochrome *bd* serves as a high oxygen affinity oxidase, allowing some pathogenic bacteria to infect O_2-poor environments (2, 3). NO is produced by macrophages to counteract microbial infections.

We previously showed that NO inhibits the turnover of cytochrome *bd* rapidly and potently, the inhibition being quickly and fully reverted upon NO depletion (4), it seems therefore of relevance to investigate how NO interacts with *bd*-type oxidases. More recently, we investigated the reaction of NO with the catalytic intermediate F of cytochrome *bd* a reaction proposed to occur at Cu_B in haem-copper oxidases (5, 6). As previously reported for mammalian cytochrome *c* oxidase, also in cytochrome *bd*, this reaction occurs with a 1:1 stoichiometry, is fast ($k = 1.2 \times 10^5$ M^{-1} s^{-1} at 20°C) and leads to a nitrite-bound form of the oxidized enzyme (7).

These results on a copper-lacking oxidase suggest that, in contrast to previous proposal, in terminal oxidases Cu_B is not essential or possibly not involved at all in the reaction of NO with the intermediate F.

1. Junemann, S (1997) *Biochim. Biophys. Acta* 1321, 107.
2. Baughn, A. D., and Malamy, M. H. (2004) *Nature* 427, 441-444
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THE FLUX CONTROL OF CYTOCHROME C OXIDASE ON CELL RESPIRATION IS CONTROLLED BY MITOCHONDRIAL $\Delta\mu H^+$ AND MEDIATED BY CARDIOLIPIN.

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Introduction

Recent measurements of the reserve capacity of cytochrome *c* oxidase on the respiratory activity in intact cells have promoted to a re-appraisal of its "*in vivo*" regulatory function. We have further extended this study in the framework of the metabolic control analysis and evaluated the impact of the mitochondrial transmembrane electrochemical potential ($\Delta\mu H^+$) on the control strength exerted by the oxidase.

Materials and Methods

Metabolic flux control analysis of endogenous respiration in cultured HepG2 , L6 and NHDF-neo cells, by cyanide titration of the integrated and isolated cytochrome c oxidase step, was carried out by respirometry. Mitochondrial membrane potential was assessed by laser scanning confocal microscopy using the dual-fluorescence emitter specific probe MitoCapture.

Results

Under respiratory state III condition (i.e. fully phosphorylating or in the presence of uncoupler) the flux control coefficient of cytochrome c oxidase over endogenous cell respiration was 0.68 ± 0.04; in the presence of mt$\Delta\Psi$ (*plus* oligomycin) or mtΔpH (*plus* oligomycin and valinomycin) the control coefficient decreased to 0.25 ±0.03. Treatment of cells with the cardiolipin ligand nonyl acridine orange (NAO) resulted in a marked decrease of the control strength exerted by cytochrome oxidase in the presence of mt$\Delta\mu H^+$, but was ineffective in the absence of mt$\Delta\mu H^+$. The change in the control coefficient, reserve capacity and threshold of an enzymatic step over a simulated multi-step metabolic flux was modelled as a function of an equilibrium between a random collisisonal- vs an aggregated-controlled state.

Conclusions

Our results show that the mitochondrial $\Delta\mu H^+$ modulates the cellular respiratory flux control exerted "*in vivo*" by cytochrome *c* oxidase. Moreover this effect might be mediated by a change in the assembly state of the OXPHOS complexes with stabilization of "respirasomes" favoured by collapse of membrane potential with cardiolipin playing a role of a sensor/transducer of the membrane energy state. Finally, our data can help in understanding the exercise-intolerance manifested in mitochondrial neuromuscular diseases and in designing new therapeutic protocols.

INVITED LECTURE

REGULATION OF MITOCHONDRIAL BIOGENESIS AND FUNCTION BY NITRIC OXIDE

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The short-lived messenger nitric oxide (NO) regulates of a variety of functions including vascular tone, immune responses, neurotransmission, cell/tissue growth differentiation and development. Most of the physiological actions of NO are mediated through activation of guanylate cyclase and generation of the second messenger cyclic GMP (cGMP), or through inhibition of cytochrome c oxidase in the mitochondrial respiratory chain. Inhibition of cytochrome c oxidase by NO has been investigated extensively and shown to play important roles in the regulation of cell respiration and oxygen sensing. Recent findings from our group show that NO regulates mitochondria also through cGMP generation. The focus of the presentation will be on how NO/cGMP regulates mitochondrial biogenesis, the mechanism of such regulation and its effects on ATP generation by the mitochondrial respiratory chain. The regulation of mitochondrial function by NO will be discussed in the context of cell/tissue energy balance and metabolism.

THE ROLE OF FLAVODIIRON PROTEINS IN NITRIC OXIDE DETOXIFICATION

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The flavodiiron proteins (FDPs) are widespread in prokaryotes (mainly anaerobic), being also present in pathogenic protozoa. FDPs are soluble modular proteins, their structural core consisting of a metallo-*β*-lactamase module, harbouring a non-heme diiron centre, fused to a FMN-binding flavodoxin module. There is growing evidence for FDPs to be part of the prokaryotic line of defence against nitric oxide (NO) derived 1) from activated macrophages as part of the mammalian host immune response to invading pathogens or 2) from denitrifying organisms inhabiting the same environment. Nitric oxide is known to impair many cellular processes in microbes by targeting key metabolic enzymes, harbouring redox active metal centres. The detoxification mechanism involving FDPs accomplishes the coupling of NADH oxidation to NO reduction, most probably to the innocuous nitrous oxide, N_2O. Remarkably, FDPs have been shown to afford *in vivo* protection against nitrosative stress. Here is presented an overview of the current knowledge on FlavoDiiron Proteins as a novel family of NO-detoxifying enzymes.

CORRELATIVE ANALYSIS BETWEEN HEMATOPOIETIC STEM CELLS DIFFERENTIATION AND MITOCHONDRIOGENESIS

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Introduction

Hematopoietic stem cells (HSCs) constitute a reservoir of undifferentiated cells that can be committed, upon appropriate stimuli, in the haematic lineages as well as in other cell types. Although residing in a bone-marrow hypoxic microenvironment (niche) and relying on an anaerobic glycolytic metabolism, ultrastructural analysis reveals in its cytoplasm the occurrence of mitochondria. The aim of this work was to characterize the poorly studied HSCs mitochondria with specific concern of their role during the early phases of commitment.

Materials and Methods

Human HSCs were obtained by bone marrow (BM-HSCs) and by G-CSF-mobilized peripheral blood (PB-HSCs). In both cases purification of HSC was achieved by immuno-selection against the specific markers CD133 and CD34. Mitochondrial activity was measured in intact cell by respirometry. Morpho-functional features of HSC-mitochondria and expression of the HSC-surface commitment markers were analyzed by confocal laser scan microscopy (CLSM) and cytofluorimetry.

Results

Measurement of oxygen consumption rate (as well as spectral and 2D electrophoretic analysis) in PB-HSCs confirmed a poor OXPHOS-phenotype. CLSM imaging of mitochondria in either PB- and BM-HSCs displayed a punctuate rather than interconnected network. Co-staining of mitochondria and CD34/CD133 stemness-markers revealed a striking inverse correlation (observed by cytofluorimetry and confirmed by CLSM). Finally HSCs produced i) DCF-detectable and DPI-inibitable reactive oxygen species (ROS) attributable to constitutive(not-stimulable) NADPH oxidase (NOX) activity and ii) DAF-detectable L-NAME-inibitable nitric oxide (NO).

Conclusions

Our results show that HSCs in the early phase of commitment undergo a progressive increase of mitochondrial mass suggestive of a bioenergetic up-regulation to suit oncoming proliferative/differentiatiative phenotype. In addition, a redox signaling, mediated by ROS/NO production and possibly triggered by changes in the environmental oxygen tension along with growth-/differentiation-factors stimulation, might be linked to mitochondriogenesis.

MELATONIN PROTECTS AGAINST MITOCHONDRIAL DYSFUNCTION ASSOCIATED WITH CARDIAC ISCHEMIA-REPERFUSION BY PREVENTING ROS-INDUCED CARDIOLIPIN OXIDATION.

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There is an ample evidence demonstrating that reactive oxygen species (ROS) play an important role in producing lethal cell injury associated to cardiac ischemia/reperfusion. Mitochondrial respiration, mainly at the level of complex I and III, is an important source of ROS generation and hence a potential contributor of cardiac reperfusion injury. Appropriate antioxidant strategies could be particularly useful to limit this ROS generation and associated mitochondrial dysfunction. Melatonin has been shown to effectively protect against ischemic-reperfusion myocardial damage. The mechanism by which melatonin exerts this cardioprotective effect is not well established. In the present study we examined the effects of melatonin on various parameters of mitochondrial bioenergetics in a Langerdoff isolated perfused rat heart model. After isolation of mitochondria from control, ischemic-reperfused and melatonin-treated ischemic-reperfused rat heart, various bioenergetic parameters were evaluated such as rates of mitochondrial oxygen consumption, complex I and complex III activity, H_2O_2 production and in addition, the degree of lipid peroxidation, cardiolipin content and cardiolipin oxidation. We found that reperfusion significantly altered all these mitochondrial parameters, while melatonin treatment had strong protective effect attenuating these alterations. This effect appears to be due, at least in part, to the preservation, by ROS attack, of the content and integrity of cardiolipin molecules, which plays a pivotal role in mitochondrial bioenergetics. Protection of mitochondrial dysfunction was associated with an improvement of post-ischemic hemodynamic function of the heart. Melatonin had also strong protective effect against oxidative alterations to complex I and III as well as to cardiolipin in isolated mitochondria.

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EXPLOITING THE EFFECT OF COMPLEX I INHIBITORS AND THE ROLE OF ENDOGENOUS/EXOGENOUS QUINONES ON ROS PRODUCTION AND ON NADH-DCIP REDUCTASE ACTIVITY.

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The molecular mechanism of catalysis of Complex I is not completely understood principally due to lack of detailed structural information: it is the only enzyme of the respiratory chain to remain a "L shaped black box". The FMN is the entry point for electrons from NADH, while N2 iron-sulfur cluster is considered to be the direct electron donor to ubiquinone. It is most likely located in the connection between the peripheral and the membrane arm suggesting that the ubiquinone headgroup could somehow reach up into the peripheral arm (Brandt et al. FEBS Lett. 2003, 545,9-17). Complex I produces reactive oxygen species (ROS): N2 was suggested to be the site of electron leak (Genova et al. FEBS Lett. 2001,505,364-368). We have studied the effect of different Complex I inhibitors on ROS production to elucidate the mechanism by which N2 transfer electrons to coenzyme Q. The working hypothesis it is that during normal redox cycle the electron leak from Complex I is very low: it can be increased by the presence of inhibitors.

Previous experiments done in our laboratory allowed us to distinguish Complex I inhibitors into two classes:

1. Rotenone-like inhibitors which increase ROS production,
2. Stigmatellin-like inhibitors which block ROS production.

Moreover water soluble quinone molecules, such as CoQ_1, in presence of Rotenone-like inhibitors exert a strong prooxidant effect that is not shared with more hydrophobic quinones such as Decylubiquinone (DB).

The above observations suggest: a) different site of interaction for the classical Complex I inhibitors, b) the monovalent reduction site for molecular oxygen should be located in a hydrophilic environment: the iron-sulphur cluster N2 might be indicated as the direct oxygen reductant.

These conclusions might be consistent with two schemes for electron pathway:

1. a linear one with the stigmatellin inhibition site located before, while the Rotenone inhibition site is located after the N2 center.
2. A bifurcate scheme in which the two electrons necessary to the complete quinone reduction come from two iron-sulphur centers one of which is responsible for semiquinone formation (Rotenone sensitive site), while the other reduces the semiquinone to the quinol form (Stigmatellin sensitive site).

To distinguish between the two proposed schemes we have attempted to study the effect of different Complex I inhibitors, and the role of endogenous and exogenous quinones on Complex I electron transfer to a water soluble electron acceptor: Dichlorophenolindophenol (DCIP). For this purpose we have used both submitochondrial particles (SMP) and beef heart mitochondria (BHM) depleted of the endogenous CoQ_{10} by pentane extraction previous lyophilization.

The results obtained suggest that the NADH-DCIP reduction pathway might pass through a semiquinone molecule that may be formed in presence of Stigmatellin-like inhibitors, but is blocked by Rotenone-like inhibitors.

SUCCINATE CONTROL OF H_2O_2 GENERATION IN MITOCHONDRIA

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Mitochondria generate $O2^-$ (which exits as H_2O_2) mostly at complex I of the respiratory chain. H_2O_2 production by NAD-linked substrates.(glutamate/malate, G/M)) is low and rotenone-stimulated, while with succinate it is very high (over 10 times faster); however, being rotenone-inhibited and potential-dependent, it is conceived as linked to a backflow of electrons from complex II to complex I. In rat brain mitochondria the H_2O_2 release as a function of succinate concentration was the half-maximal at 0.35 mM. The same titration performed in the presence of G/M (1 or 2 mM each) which per se produced only minimal amount of peroxide did not modify the extent of succinate stimulation but only shifted the half maximal effect of succinate to 0.5 or 0,6-0,7 mM with 1 or 2 mM G/M respectively. The overall succinate stimulation was the lowest in the absence of ΔpH (i.e. in the presence of Na^+/H^+ exchanger nigericin) and highest at high ΔpH (i.e. in the absence of Pi). To show that G/M are still oxidized in the presence of succinate we monitored respiration rates with G/M (1 mM) succinate or both.

O_2 consumption was higher with succinate than with G/M, and was intermediate with the mixture, an indication that G/M are indeed oxidized in the presence of succinate. Above 1,2 mM succinate the effect of G/M in slowing the succinate respiration rate disappeared. The results indicate that 0,2-0,5 mM succinate (reported to be the physiological concentration range in the cell) may control H_2O_2 release at complex I during oxidation of NAD-linked substrates.

The described modulation of H_2O_2 production has been found essentially similar in mitochondria from heart and muscle. Moreover in the heart and muscle mitochondria, the succinate stimulation of H_2O_2 release is also negatively controlled by palmitoyl-CoA (but not by shorter CoA esters). Also palmitate or oleate are effective inhibitors but only in the presence of CoASH and ATP, thus presumably forming long chain CoA-thioesters. In the case of palmitoyl-CoA the half inhibition is obtained at about 10 μM, a concentration conceivably having a physiological meaning.

EFFECTS OF INHIBITORS OF NAD(P)H OXIDASE ON GLUCOSE TRANSPORT ACTIVITY IN A LEUKAEMIC CELL LINE

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The NADPH oxidase and its role in the cellular immune response of phagocytic white blood cells have been well described: in phagocytes the enzyme is composed of at least the four main subunits, gp91phox, p22phox, p47phox and Rac1 which are all necessary for the complex to transfer electrons from NADPH, via FAD and cytochrome, to oxygen to generate superoxide. In more recent years, the generation of low levels of superoxide by a range of different non-phagocytic cells has been attributed to the presence of a NAD(P)H oxidase-like enzymes (NOX family) in these cells, reinforcing the idea that ROS are "intentionally" generated, in various cell types, with biological functions [1].

We have recently shown [2,3] that, in B1647 cell line - established from bone marrow cells of a patient with acute myelogenous leukaemia - there is a correlation between ROS and an important physiological activity, such as glucose uptake, which is up-regulated in leukaemic cells. By using antioxidants, generation of ROS was shown to be crucially involved in the modulation of glucose uptake mediated by Glut1. Therefore, we tried to elucidate the sources of ROS generation and the mechanisms by which ROS are involved in the regulation of Glut1 activity.

Data obtained in the presence of NAD(P)H oxidase inhibitors suggest that a possible ROS generation site could be this membrane-bound enzymatic complex, similar to the phagocytic one.

Although large-scale studies have not yet been carried out, early data indicate that overexpression of NOX enzymes is a frequently observed feature of cancer cells, consistent with the earlier observation of increased ROS levels in cancer cells [4]. These data indicate that NOX enzymes might have a role in the tumour abnormal growth and that they could be a new target for cancer chemotherapy and treatment of other conditions related to aberrant cell growth [1].

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MITOCHONDRIAL OXIDATIVE METABOLISM IN INTERLEUKIN 7-ENGINEERED STROMAL CELLS

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Introduction

Human stromal cells engineered with a retroviral vector carrying the interleukin 7 (IL7) gene are used for cotransplantation with CD34+ stem cells to improve the immunological reconstitution in T cell-depleted hosts, since IL-7 is a cytokine playing a pivotal role in human T-cell survival , development and homeostasis. Transduced stromal cells in vivo home to lymphoid organs and produce sufficient IL-7 in loco, supporting T cell development, but the effect is not long-persisting. In this study we analysed the mitochondrial oxidative metabolism in IL7-stromal cells to reveal possible alterations influencing the cell survival.

Materials and Methods

An analysis of the OXPHOS complexes activity and related ROS production was carried out in IL 7-stromal cells and compared to normal stromal cells.

Results

The endogenous respiration as well as the complex I activity resulted to be significantly reduced in engineered cells compared to the control; confocal microscopy revealed an increase of mitochondrial ROS production whereas the mitochondrial morphology was not effected. The antioxidant transcript levels measured by RT-PCR were not modified. A dibutyryl c-AMP treatment resulted in disappearance of ROS and rescue of the complex I activity.

Conclusions

Engineered stromal cells show a reduced complex I activity responsible of ROS production completely reversed by dcAMP treatment; this could explain the partial unsuccess of the transplantation protocol and provides insights to improve the efficiency of the engineered stromal cell therapy in regenerative medicine.

THE DIFFERENTIATION PROCESS OF HUMAN MONOCYTE-DERIVED DENDRITIC CELLS: POSSIBLE ROLE OF MITOCHONDRIA AND REACTIVE OXYGEN SPECIES

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Dendritic cells (DC) are potent antigen presenting cells (APC) capable to induce T and B cell response and immune tolerance (1). DC derive from bone marrow precursors and reside in an immature state in peripheral tissues where they exert a sentinel function for incoming antigens (Ag). Following an encounter with an Ag, DC undergo a maturation process that enhances their APC function and promotes their migration to the draining lymph nodes where they present processed Ag to naïve T cells. Recent studies suggest a role for Reactive Oxygen Species (ROS) as essential second messengers for DC response to several physiological stimuli (2). It is well established that the basal production of cellular ROS is mainly associated to the mitochondrial electron transport chain activity. Two sites of the respiratory chain, namely complex I and complex III, have been suggested to be the major source of ROS.

We are currently investigating the role of mitochondrial oxidative phosphorylation system in the differentiation and maturation processes of human DC generated from immuno-magnetically selected CD14+-monocytes.

Our preliminary results show that i) the differentiation process of dendritic cells is characterised by an increase of the endogenous respiration, and ii) the presence of sub-saturating concentrations of the complex I inhibitor rotenone (180 nM) inhibits DC differentiation process without affecting cell viability. Accordingly, rotenone-treated cells are characterised by an increased expression of CD14 (monocyte marker), a decreased expression of CD1a (marker of DC differentiation), and by the presence of CCR7, a chemokine receptor involved in the trafficking and homing of DC to secondary lymphoid organs; iii) cells cultured in the presence of rotenone exhibit lower ROS content as compared to control cells. Given the strategic localization of DC at the interface of innate and adaptive immunity, this study may provide the rational for the identification of new targets in the regulation of DC functions.

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INVITED LECTURE

PARAPLEGIN-AFG3L2 COMPLEX, A LARGE MITOCHONDRIAL METALLOPROTEASE WITH A ROLE IN NEURODEGENERATION AND NOT ONLY

Giorgio Casari

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GENERATION OF A TRANSGENIC MOUSE MODEL OVEREXPRESSING OPA1, A MITOCHONDRIA-SHAPING PROTEIN MUTATED IN DOMINANT OPTIC ATROPHY

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OPA1 is a dynamin-related protein of the inner mitochondrial membrane, mutated in dominant optic atrophy. OPA1 promotes mitochondrial fusion cooperating with MFN1, a protein of the same family located in the outer mitochondrial membrane. Moreover, our recent results point to a role for OPA1 in the apoptotic remodelling of mitochondrial cristae, by keeping in check the narrow tubular cristae junctions. In order to dissect the role of cristae remodelling and mitochondrial fusion sustained by OPA1 in vivo, we generated a transgenic mouse overexpressing OPA1. The OPA1 cDNA was cloned under the control of the ubiquitous human β–actin promoter and the transgene was inserted by homologous recombination upstream of the X-linked *Hprt* locus. Chimeras were born viable; they are fertile and show a strikingly accelerated growth, weighing 30% more than their littermates. We will present data on the initial physiological and molecular analysis of the mitochondrial phenotypes of these animals.

OPA1 CONTROLS APOPTOTIC CRISTAE REMODELLING INDEPENDENTLY FROM MITOCHONDRIAL FUSION

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Mitochondria amplify activation of caspases during apoptosis by releasing cytochrome c and other cofactors. This is accompanied by fragmentation of the organelle and remodeling of the cristae. Molecular mechanisms governing the latter remain unclear. Optic Atrophy 1 (OPA1), a pro-fusion dynamin-related protein of the inner mitochondrial membrane mutated in dominant optic atrophy protects from apoptosis by preventing cytochrome c release. This is independent from mitochondrial fusion but depends on the oligomerization of two forms of OPA1, the soluble, intermembrane space and the inner membrane integral one. The pro-apoptotic BCL-2 family member BID disrupts OPA1 oligomers, while high levels of OPA1 stabilize them and prevent mobilization of cytochrome c. OPA1 does not interfere with activation of the mitochondrial “gatekeepers” BAX and BAK, but controls shape of mitochondrial cristae during apoptosis. Thus, OPA1 has genetically and molecularly distinct functions in mitochondrial fusion and in cristae remodelling during apoptosis.

MITOCHONDRIAL RHOMBOID PARL REGULATES CYTOCHROME C RELEASE DURING APOPTOSIS VIA OPA1-DEPENDENT CRISTAE REMODELING.

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Rhomboids, evolutionarily conserved integral membrane proteases, participate in crucial signaling pathways. Presenilin associated rhomboid like (PARL) is an inner mitochondrial membrane rhomboid of unknown function, whose yeast orthologue is involved in mitochondrial fusion. Parl$^{-/-}$ mice display normal intrauterine development, but from the 4th postnatal week undergo progressive multisystemic atrophy leading to cachectic death. Atrophy is sustained by increased apoptosis, both in and ex vivo. Parl-/- cells display normal mitochondrial morphology and function, but are no longer protected against intrinsic apoptotic death stimuli by the dynamin-related mitochondrial protein OPA1. Parl$^{-/-}$ mitochondria display reduced levels of a soluble, intermembrane space (IMS) form of OPA1 and OPA1 specifically targeted to IMS complements Parl$^{-/-}$ cells, substantiating the importance of PARL in OPA1 processing. Parl-/- mitochondria undergo faster apoptotic cristae remodeling and cytochrome c release. These findings implicate regulated intramembrane proteolysis in controlling apoptosis.

ENERGETIC EFFICIENCY AND MITOCHONDRIAL FUSION IN FIBROBLASTS FROM DOMINANT OPTIC ATROPHY PATIENTS BEARING THE C.2708DELTTAG OPA1 MUTATION

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The Kjer syndrome or autosomal Dominant Optic Atrophy (DOA) is the most common mitochondrial neuropathy, due to a selective degeneration of retinal ganglion cells, leading to optic atrophy and blindness. A subset of DOA cases has been associated with heterozygous mutations in the OPA1 gene located on chromosome 3q 28-29 [1, 2]. This gene encodes for a dynamin-related GTPase, OPA1, which is targeted to mitochondria by an N-terminus import sequence motif and is anchored mainly to the inner membrane facing the intermembrane space [3]. The most common OPA1 mutation found in DOA is the c.2708delTTAG deletion localized at the C-terminal coiled-coil domain of the protein. In the present study we have investigated the energetic efficiency of skin fibroblasts derived from DOA patients bearing the c.2708delTTAG deletion using the galactose model, i.e. forcing the cells to use solely the respiratory function for energy production. Under these experimental conditions, DOA fibroblasts could maintain normal ATP levels and proliferate, suggesting that the energetic function is not significantly impaired. However, analysis of mitochondrial morphology revealed that mitochondria form a tubular structure similar in DOA and control fibroblasts in glucose medium, whereas after 24h incubation in galactose medium a significant percentage of DOA fibroblasts only exhibited a network with balloon-like structures. Using a photoactivable, mitochondria-targeted GFP (4), the rate of mitochondrial fusion was shown to be slightly increased in DOA fibroblasts incubated in glucose, whereas it was completely inhibited in cells exhibiting balloon-like mitochondrial structures.
In conclusion, the results indicate that the mitochondrial fusion of fibroblasts bearing the c.2708delTTAG OPA1 deletion is surprisingly similar to controls under glycolytic conditions, whereas it is strongly reduced when cells are forced to utilize an oxidative metabolism. Thus, forcing the respiratory function unravels a failure in the mitochondrial network dynamics of DOA fibroblasts.

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FUNCTIONAL AND MOLECULAR RESPONSES TO STRESS CONDITIONS IN HETEROPLASMIC MELAS 3243 RD CYBRIDS.

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Clinical status of human mitochondrial disorders associated with heteroplasmic mtDNA mutations is greatly dependent on the residual amount of wild type mtDNA molecules. No clinical symptoms or biochemical respiratory chain defects are detected above a relatively low threshold of wild-type mtDNA proportion. Therapeutic approaches to human disorders are therefore trying to modify the relative proportion of wild- type and mutant mtDNA. In that prospect a better understanding of conditions and mechanism by which cells modify their mtDNA mutant amount by a segregation from heteroplasmic to homoplasmic status or by increasing proportion of wild-type mtDNA molecules, could help us better understand the pathogenesis of mitochondrial diseases and could deserve potential consequences regarding therapeutic approaches to human diseases associated with heteroplasmic mtDNA mutations.

Mitochondrial Encephalopathy Lactic Acidosis and Strocke (MELAS) syndrome is due to various point mutations in the gene encoding tRNA $^{LEU(UUR)}$. The most frequent and studied mutation is at 3243 bp. To detect potential conditions that will favour changes in percentage of heteroplasmy, different heteroplasmic cytoplasmic hybrids (cybrids) for 3243 MELAS mutation in muscle nuclear background (MELAS RD cybrids) have been established. 99%, 91% and 80% and 0% mutant MELAS RD cybrids were cultured in medium containing different amount of glucose and uridine. RD cybrids were monitored for the following physiological and molecular parameters: growth rate expressed as duplication time, O2 consumption, percentage of mutant mtDNA and quantification of mtDNA amount by RT- PCR.

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COMPARATIVE BIOENERGETIC STUDIES OF CELLS CARRYING THE 8993T>G OR 8993T>C MITOCHONDRIAL DNA MUTATIONS

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The 8993T>G and T>C mutations of the mitochondrial genome change the highly conserved Leu-156 in the ATPase 6 subunit of the F1F0-ATPase (ATP synthase), to Arg or Pro, respectively. These mutations have dramatic consequences for individuals: the clinical phenotype associated with both mutations depends on the mutation load (or heteroplasmy), being the symptoms milder and presenting a later onset when the T>C change occurs. Thus, the T>G and T>C at heteroplasmy above 70% are considered pathogenic for Neurogenic muscle weakness, Ataxia, and Retinitis Pigmentosa (NARP) or Leigh syndromes (Di Mauro and Davidzon, 2005; Lenaz et al. 2004). In the last decade a number of studies have been reported on the T>G mutation, whereas the rarer T>C has been only poorly investigated. The present work reports data recently collected in our laboratory where as many as 10 lymphocytes samples from individuals carrying the T>G mutation and two T>C samples have been studied. ATP synthesis catalyzed by ATP synthase decreases by a not statistically significant 18% in lymphocytes of the 8993T>C patient carrying an almost homoplasmic mutation, it is not affect the NARP patient's mother carrying 9% mutation load, and it decreases sharply in lymphocytes of five individuals harbouring the 8993T>G mutation, according with results previously reported (Sgarbi et al. 2006). Under state 3 respiratory conditions a slightly higher level of mitochondrial $\Delta\psi_m$ was observed, reaching statistical significance only in T>C mutant lymphocytes carrying as high as 93 % mutation load. This behaviour is similar to that observed in the T>G mutant lymphocytes. Moreover, oligomycin enhanced mitochondrial $\Delta\psi_m$ to a much higher extent than the mutations, therefore ruling out that the two mutations at nt8993 could block proton transport through the F1F0-ATPase, but suggesting a decreased rate of proton translocation by the enzyme (manuscript in preparation). These findings will be discussed in relation with the possible mechanism of dysfunction caused by the mutations.

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NEW INSIGHTS IN THE PATHOPHYSIOLOGY OF RIBOFLAVIN-RESPONSIVE MULTIPLE ACYL-COA DEHYDROGENASE DEFICIENCY (RR-MAD).

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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) β-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 biochemical and molecular tests to better characterise the metabolic disturbance of this disease and to explore the pathophysiological events (1-3). Biochemical alterations of pre-therapy patient muscle samples included reduction in FA β-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. The reduced activity was often associated with a reduction in flavoenzyme amount, as quantified by Western blotting. In the pre-treatment samples, FMN and 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 (4-5). These metabolic alterations were associated with an increase in uncoupling protein 3 (UCP3) mRNA and protein amount (2). Muscle UCP3 is up-regulated under conditions that either increase the levels of circulating free FA and/or decrease FA β-oxidation. We postulated that the up-regulation of UCP3 in RR-MAD is due to the accumulation of muscle FA/acyl-CoA. All the altered parameters were restored to control values after riboflavin treatment. Proteomic investigation of muscle mitochondria in one RR-MAD patient (3) revealed decrease or absence of several proteins belonging to different groups: 1) flavoenzymes or subunits of flavin-dependent enzymes, such as electron transfer flavoprotein-ubiquinone oxidoreductase and NADH-ubiquinone oxidoreductase 75kDa subunit; 2) enzymes related to flavin cofactor-dependent mitochondrial pathways, such as trifunctional enzyme beta subunit; 3) mitochondrial or mitochondrial associated calcium binding proteins. All these deficiencies were completely rescued by riboflavin treatment. Proteomic data indicate that the enzymatic defects are multiple, coordinated, and riboflavin-responsive. The mRNAs amounts of certain proteins, quantified by Real-time-PCR, were reduced in pre-therapy samples and returned to normal values after riboflavin treatment. Taken together, these results indicate for the first time a profound involvement of riboflavin/flavin cofactors in modulating the expression of a number of functionally coordinated polypeptides involved in fatty acyl-CoA and amino acid metabolism.

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MEGACARIOBLASTIC LEUKEMIA AND MITOCHONDRIA: A CASE REPORT

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Introduction
Recent investigations have suggested a possible role of mitochondrial dysfunctions in myelodisplastic and acute myeloid leukaemia. In this study we have carried out functional analysis and mtDNA sequencing of a rare haematological malignancy.

Materials and Methods
Peripheral blood cells collected by a patient affected by megacarioblastic leukaemia were analysed for functional /structural features of the mitochondrial respiratory activity and their mtDNA sequenced.

Results
Systematic measurements of respiration in intact cells revealed very low activity and abnormal responces to canonical inhibitors of the mitochondrial respiratory chain. Bidimensional BN-PAGE of mitoplast protein extracts displayed differences in the polypeptide patterns with respect to control mitochondria. Finally extensive sequencing of the mtDNA unveiled the occurrence of two omoplasmic mutations in the ND1 gene. One of these mutations was already described in mitochondria-related encephalopaties, the other (A3418G, Asn $\rightarrow$ Asp) was never reported before. Both mutations are in a highly conserved hydrophilic loop exposed to the mitochondrial inner phase and suggested to be the binding site for rotenone/ubiquinone

Conclusions
Our results strongly support the monoclonal origin of this type of myeloid acute leukaemia possibly derived by deregulated proliferative activity of a megacariocyte precursor and in addition suggest the possible involvement of mitochondrial genetic alterations with related defects in the OXPHOS system as a co-cause in the development of the disease.

INVITED LECTURE

REGULATED EXO-ENDOCYTOSIS, AN EXPANDING WORLD: PROPERTIES AND ROLE OF A NEW EXOCYTIC ORGANELLE, THE ENLARGEOSOME.

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Regulated exocytosis, the triggered fusion of the membrane of intracellular organelles with the plasma membrane, is most often confused with regulated secretion, a process based on exocytosis which however includes also the release of the vesicle/organelle content. However, exocytosis does not result only in the release of the content but also in the incorporation at the cell surface of the membrane delimiting the exocytic organelles. Tenth of papers, dealing with a variety of scientific problems, have documented that the traffic to the cell surface of key proteins (receptors, transporters, channels) can occur by regulated exocytosis apparently occurring without any secretion. Other non-secretory exocytoses are aimed at the enlargement of the plasma membrane during processes such as cytodieresis, phagocytosis, neurite growth and wound healing. Non-secretory exocytoses are therefore as important as their secretory counterparts (see Chieregatti and Meldolesi, Nature Rev. Mol. Cell Biol., 2005). Among the non-secretory exocytic organelles are the enlargeosomes, discovered in our lab in 2002 while working on a secretion-defective clone of PC12 cells, and then reported in several other types of cell.

Enlargeosomes are mostly located in the proximity of the plasma membrane. Their name comes from their participation in many membrane enlargement processes, apparently without any content release. Enlargeosomes have been shown to participate in wound healing and nerve cell differentiation, and to account for the process that compensates the surface loss induced by macropinocytosis in dendritic cells. Recently, the ultrastructure of these organelles, as well as their exocytosis in response to stimulation, have been revealed by high resolution immunocytochemistry. Enlargeosomes are small vesicles (~60nm in diameter) and their membrane is coated on both sides: on its internal surface by a large protein, Ahnak; on its cytosolic surface by annexin2, a cytosolic protein that binds also to other membranes, however only in response to $[Ca^{2+}]_i$ increases. On the enlargeosome membrane annexin2 coating is unique because it is stable also at rest and is necessary for the vesicle to undergo exocytosis. Recycling of the exocytized enlargeosome membranes occurs by a peculiar, non-clathrin-dependent process that appears distinct also from the numerous non-acidic forms of the process described in the last very few years. In addition to endocytosis, the enlargement of the plasma membrane due to enlargeosome exocytosis can be compensated by specific vesicle shedding. In my presentation I will report on new properties of enlargeosomes and of their endocytic vesicles that we have identified during the last few months.

FUNCTIONAL AND STRUCTURAL CHARACTERIZATION OF THE N-TERMINAL PEPTIDE OF THE MITOCHONDRIAL VDAC1.

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VDAC or mitochondrial porin is the most abundant transmembrane protein in the mitochondrial outer membrane. Bacterial porins are formed of a typical β-barrel structure with almost any α-helix. The mitochondrial VDAC structure has been also predicted to form a β-barrel [1]. Interestingly its N-terminal domain has been predicted as an amphipathic α-helix [1]. It is debated whether this putative α-helix crosses the membrane and whether it has a relevant function as a voltage sensing region. To elucidate both the conformational behaviour and the function of this region, the 19 residues peptide fragment Ac-AVPPTYADLGKSARDVFTK-NH_2 from the VDAC protein's N-terminal region, has been synthesised. The secondary structure preferences of this peptide have been established by means of CD and NMR spectroscopy under different experimental conditions (pH, solvents, temperature, micellar environment, etc.). Moreover DSC (Differential Scan Calorimetry) measurements were carried out to evaluate the membrane affinity of this synthetic peptide. The structure of the peptide in SDS has been resolved by NMR. The protein lacking the same N-terminal peptide has been expressed in eukaryotic cell. The truncated form of VDAC1 is targeted to mitochondria, indicating that this sequence is not involved in this process. The recombinant protein expressed in vitro, refolded and reconstituted in artificial membrane system shows a disturbed channel forming activity. The overall results indicate a critical role of the N-terminal moiety in the functional features of VDAC1.

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DYNAMIC ASPECTS OF THE MITOCHONDRIAL CARNITINE/ACYLCARNITINE CARRIER STRUCTURE, AS REVEALED BY CHEMICAL MODIFICATION OF FOUR REPLACEMENT CYS MUTANTS.

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The carnitine/acylcarnitine carrier (CAC), that is responsible for the transfer of acylcarnitines into the mitochondrial matrix, belongs to the mitochondrial carrier protein family. As the other members of the family, the CAC has six transmembrane α-helices connected by hydrophilic segments on the matrix and the cytosolic side (1). To study the structure-function relationships by site-directed mutagenesis, the CAC has been over-expressed in *E. coli*; the recombinant WT or mutant proteins have been reconstituted into liposomes and the transport assayed as ^{3}H-carnitine/carnitine antiport. The role of Cys residues of the CAC have been investigated in single and multiple mutants, in which one or more of the six native Cys residues have been substituted with Ser. Analysis of transport activity inhibition by mono-functional SH reagents revealed that C136 is the major target for SH reagents and is located at or near the substrate binding site (2). More recently, by using SH oxidizing, cross-linking and coordinating reagents it has been demonstrated that C58, C136 and C155 are or became close each other during some steps of the transport pathway (3). To gain insight in the conformational changes of the protein associated with the translocation of the substrate, CAC mutants containing only two of the vicinal Cys have been constructed. The reconstituted CAC mutants, as the native protein, function according to the ping-pong mechanism, that implies one substrate binding site alternatively exposed to the external (outward facing conformation) or to the internal (inward facing conformation) side of the carrier. According with this mechanism, the conformation of the proteins has been varied using either low (2.5mM) or high (15mM) intraliposomal carnitine concentration; the last condition favours the inward facing conformation. The inhibition of the carnitine antiport by diamide, caused by the formation of an S-S bridge between vicinal Cys residues, was greater in the presence of the higher intraliposomal carnitine concentration in the mutants containing C58 and C136 (C23/89/155/283S) or C58 and C155 (C23/89/136/283S); whereas the inhibition of the mutant containing C136 and C155 (C23/58/89/289S) did not depend on the carnitine concentration. Hydrophilic SH reagents MTSES, MTSEA and fluorescein maleimide, externally added to the proteoliposomes, did not inhibit the five replacement mutant C23/89/136/ 155/283S, indicating that C58 is not accessible to the reagents. In contrast, the four replacement mutants C23/89/155/283S and C23/89/136/283S were inhibited by these reagents. The inhibition was more efficient at higher intraliposomal carnitine concentration, indicating that the inward conformation of the protein facilitated the access of the reagents to C136 and C155. The experimental results can be explained by movements of at least three domains of the CAC: the transmembrane α-helix III that contains C136 and the matrix hydrophilic segments M1 and M2 that contain C58 and C155, respectively. The results can be interpreted in the light of the homology model of CAC constructed using the X-ray structure of the ADP/ATP carrier (4) as template.

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BIOTIN-TAGGING OF BILITRANSLOCASE IN INTACT RAT LIVER AS A MEAN FOR ITS DETECTION AND PURIFICATION BY AVIDIN-BIOTIN TECHNIQUES

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Bilitranslocase (T.C. 2.A.65.1.1) is a liver membrane transport protein responsible for the translocation of several organic anions into the hepatocyte. Both experimental evidence and computational modelling suggest that some domains of bilitranslocase protrude outside the membrane into the vascular compartment. Some additional evidence suggests that bilitranslocase exposes some reactive cysteine(s) outside the membrane. Such properties of bilitranslocase were exploited to biotin-tagging bilitranslocase by means of a sulphydryl-specific reagent carrying a biotin moiety, such as N-biotin-maleimide, while restricting the range of other biotin-tagged proteins to the sinusoidal domain of the hepatocyte plasma membrane.

Some additional evidence suggests that bilitranslocase is an allosteric protein, whose high-vs. low-affinity state is regulated by the [NADH]/[NAD^+] ratio. In physiological conditions the ratio value is 1:700, and the low-affinity state of bilitranslocase is therefore expected to prevail; on the other hand, should that ratio be increased, as under various pathophysiological conditions, the protein would shift to its high-affinity state. It has been shown that the low-affinity conformation of bilitranslocase is characterised by the exposure of (a) reactive cysteine(s). Such property was exploited to further optimise the extent of biotin-tagging of bilitranslocase, while lessening the extent of biotin-tagging of other proteins on the sinusoidal domain of the hepatocyte plasma membrane.

A biotynilation procedure was set up, consisting of perfusing the rat liver with solutions containing N-biotin-maleimide while managing the [NADH]/[NAD^+] ratio. Both the efficiency and the specificity of this procedure have been evaluated by comparing avidin- and immunoblotting. As a consequence we are now able to detect and purify bilitranslocase by avidin-biotin techniques.

EXPRESSION AND FUNCTION OF BILITRANSLOCASE IN VASCULAR ENDOTHELIAL CELLS

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Bilitranslocase (T.C.2.A.65.1.1) is a liver membrane transport protein responsible for the translocation of several organic anions into the hepatocyte. A recent immunohistochemical investigation of both rat and human liver indicated that an anti-sequence anti-bilitranslocase antibody can react with some epitope expressed at the level of the vascular endothelium of hepatic arterioles. To further assess this finding, rat aorta was examined by immunohistochemistry analysis and was found clearly positive on the endothelial intima. The investigation was therefore extended to a permanent human cell line expressing highly differentiated functions characteristic of human vascular endothelium (EA.hy 926). Both immunoblotting and immunocytochemical tests revealed that those cells indeed express bilitranslocase. Transport studies using bromosulfophthalein (BSP), a cholephilic dye which is the reference transport substrate of bilitranslocase, indicated that this dye can be taken up into EA.hy 926 cells. BSP transport was found then to be inhibited by anti-bilitranslocase antibodies and by some typical substrates of bilitranslocase, such as bilirubin and nicotinic acid. Further assays were carried out using quercetin as a substrate; this flavonoid is a competitive inhibitor of bilitranslocase transport activity in rat liver plasma membrane vesicles. Quercetin uptake into EA.hy 926 cells was found to be partly dependent on the activity of bilitranslocase, therefore suggesting that the expression and function of this carrier in the vascular endothelium is connected to the bioavailability of flavonoids in such district, which in turn is believed to be important both for the protection of vessels from atherosclerosis and in the modulation of the vascular tone.

cAMP-DEPENDENT PROTEIN KINASE AND A-KINASE ANCHOR PROTEINS IN THE INNER COMPARTMENT OF MAMMALIAN HEART MITOCHONDRIA

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In mammalian cells cAMP is produced by the adenylyl cyclase of the plasma membrane and by the bicarbonate activated soluble adenylyl cyclase localized in various subcellular compartment (1). cAMP-dependent protein kinase (PKA) is considered the main effector of cAMP action. The molecular basis for distinct subcellular localization of cAMP effectors is exemplified by the class of AKAPs proteins (2). PKA has been detected in the outer and inner membrane/matrix fraction of mammalian mitochondria where it phosphorylates various mitochondrial proteins (3).

Evidence showing the existence in the inner compartment of rat-heart mitochondria of PKA and AKAP proteins is presented. Immunoblotting analysis shows that 90% or more of mitochondrial PKA and AKAP 121 is localized in the inner mitochondrial compartment. This localization is further verified by measurement of the specific catalytic activity of PKA and by radiolabelling of regulatory subunit and A-kinase anchor protein with ^{32}P-cAMP and ^{32}P-phosphorylated regulatory subunit respectively. Transmission electron microscopy of mitochondria exposed to gold-conjugated A-kinase anchor protein 121 antibody shows labelled spots of AKAP protein in the inner mitochondrial compartment apparently associated with cristae. A down-stream extension of the effect of cAMP in the inner mitochondrial compartment mediated by adenylyl cyclase, phosphodiesterases, PKA, protein phosphatase(s), EPAC proteins and G proteins can have definite impact on cellular energy metabolism, apoptosis, cell growth and differentiation.

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THE ENERGETIC FAILURE CAUSED BY SEVERE MUTATIONS IN THE MITOCHONDRIAL GENOME IS NOT RESCUED BY BCL-2 OVEREXPRESSION

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The proto-oncogene Bcl-2 prevents apoptosis and some forms of necrosis, although its exact mechanism of action is not well defined. Bcl-2 has been shown to localise to multiple cell compartments, including the outer mitochondrial membrane, the endoplasmic reticulum and the nucleus. Accumulating evidence indicates that rather than antagonize specific steps of the apoptotic pathway, Bcl-2 could regulate mitochondrial metabolism, including adenine nucleotide exchange (1) and permeability to metabolic anions (2). Recently, Bcl-2 overexpression has been shown to significantly improve oxidative phosphorylation in cells bearing pathogenic mutations in mitochondrial DNA (mtDNA) affecting the tRNA genes, which cause a severe energetic failure (3).

We have recently demonstrated that the cell line XTC.UC1, derived from an oxyphilic thyroid carcinoma, was unable to survive in galactose medium, a condition which forces cells to rely solely on mitochondria for ATP production. Furthermore, the rate of mitochondrial ATP synthesis driven by complex I substrates, but not by complex II substrates, was greatly reduced in XTC.UC1 cells. Complete sequencing of the mtDNA of these cells revealed two new mutations: an homoplasmic frameshift mutation in ND1 and an heteroplasmic non-conservative substitution in cytochrome b (4)

We decided to analyze the effect of Bcl-2 on the survival and energetic competence of these cells.

When Bcl-2 overexpressing cells were incubated in galactose medium, they appeared quite healthy upon microscopic inspection. However, both cell viability and ATP content were dramatically reduced in comparison to control cells. Accordingly, the rate of ATP synthesis driven by glutamate and pyruvate was also decreased. Finally, Bcl-2 overexpression was shown to prevent the extreme proteolytic cleavage of some cytoskeletal and cytosolic proteins observed during incubation in galactose.

In conclusion, Bcl-2 overexpression safeguards cellular adhesion and morphology, but is not able to rescue the energetic dysfunction of XTC.UC1 cells. It is likely that the main target of Bcl-2 may be the cytoskeleton/adhesion machinery.

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NASH INDUCES EXPRESSION OF UCP2 AND MITOCHONDRIA RESPIRATORY IMPAIRMENT IN LIVER HEPATOCYTES

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Introduction

Nonalcoholic Steatohepatitis (NASH) is a liver disease strictly associated with metabolic disorders like diabetes and insulin-resistance. Its etiology has not yet been explained, and its mechanisms are not well understood. Many authors agree with the "double hit" theory, according to which on a steatotic liver (first hit) a second damage occurs giving rise to necrosis, inflammation and fibrosis. Mitochondria play a key role in NASH development as the main site of fatty acids oxidation and ROS production. We investigated alterations in mitochondrial biomembrane and bioenergetic during NASH progression.

Methods

Male Wistar rats assumed High–Fat Methionine and Coline deficient diet for 4 wks. NASH was confirmed by aminotransferases and histology. Liver mitochondria analysis integrated oxygen consumption by Clark electrode and mitochondrial membrane potential by TPP+ electrode. Liver UCP-2 expression was also evaluated by real time RT-PCR.

Results

Respiratory Control Index (RCI) from Succinate was significantly higher in NASH than in control rats, as well as Complex II and IV activity. P/O ratio from Succinate was significantly lower in NASH than in control rats. In addition, mitochondria from NASH liver rats showed an increased rate of proton leak when the rate of electron transport was suppressed. UCP-2 expression was also evaluated and revealed a significant expression of steady state m-Rna expression compared to control rats.

Conclusion

Our data show that, during NASH development, mitochondria respiratory chain impairment occur probably as consequence of uncoupling agent expression. We suggest that the excessive storage of fat induces mitochondria metabolism alteration to counteract the unnecessary substrates storage in a highly redox state.

ROLE OF CALCIUM IN MITOCHONDRIAL DISFUNCTIONS INDUCED BY HEPATITIS C VIRUS INFECTION

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Introduction

It was recently shown that a proportion of hepatitis C virus (HCV) core protein expressed in hepatoma cells localizes to mitochondria, suggesting that mitochondria may be involved in HCV infection. We have extended these observations by investigating the mitochondrial oxidative metabolism in U2-OS human osteosarcoma-derived cell lines inducibly expressing the entire HCV open reading frame.

Materials and Methods

A comparative analysis of the OXPHOS complexes activity and related ROS production along with mitochondrial morpho-functional and intracellular calcium confocal microscopy imaging was carried out in U2-OS cells inducibly expressing the HCV polyprotein under the control of a tetracycline-regulated gene expression system. In these cells viral protein expression is repressed in the presence of tetracycline whereas viral proteins become detectable 6 h and reach a steady-state 24-48 h following tetracycline withdrawal.

Results

Confocal microscopy analysis using specific probes revealed that cells expressing HCV proteins exhibited: i) marked $\Delta\Psi_{mt}$ depolarisation; ii) increase of mitochondrial ROS production; iii) higher intramitochondrial calcium levels. Moreover, complex I activity (as well as the endogenous respiration) was significantly reduced whereas complexes III and IV activities were not affected. The total amount of ATP was significantly higher in HCV positive cells. Of note, all the alterations observed were rescued to the control levels upon treatment of the infected cells with calcium-uniporter inhibitors.

Conclusions

Our study shows a detrimental effect of HCV proteins on the cell oxidative metabolism with inhibition of respiratory chain activity and increased production of reactive oxygen species. These alterations come together with de-regulation of the calcium recycling between cytoplasm and intracellular Ca^{++} stores. The link appears to be causative since all the modifications observed in U2-OS cells expressing the HCV were completely reversed by the Ca^{++} uniporter inhibitor ruthenium red These results, obtained in a well-defined and reproducible cellular context, provide new insights into a possible involvement of mitochondrial dysfunction in the pathogenesis of hepatitis C and suggest alternative approaches for therapeutic strategy.

SPECIES-SPECIFIC STIMULATION OF THE MITOCHONDRIAL PERMEABILITY TRANSITION BY NORBORMIDE ANALOGUES

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It has been previously shown that norbormide (NRB), which is selectively toxic in rats, also causes opening of the mitochondrial permeability transition pore (PTP) in rats but not in mice or guinea pigs[1]. NRB is a mixture of eight racemic diastereoisomers, only three of which are lethal to rats. In order to elucidate whether species-specific toxicity and PTP induction are causally linked, in the present study we investigated the pore-regulatory properties of both lethal and non-lethal NRB isomers by following Ca^{2+} retention capacity and matrix swelling of rat, mouse and guinea pig liver mitochondria. All NRB isomers tested exhibited similar, rat-specific effects on mitochondrial permeabilization, which indicates that: (i) species-specificity of NRB action on PTP is not related to the molecular isomerism; (ii) regulation of pore activity and lethality are unrelated phenomena. A series of NRB-related neutral and cationic analogues were prepared based on the "pseudo-deconstruction" of the parent molecule, and the structural features responsible for activation of the PT were investigated. These studies demonstrated that the key moiety responsible for the pore-activating effects is represented by a 2-(phenylvinyl)pyridine group. Cationic norbormide derivatives that accumulate inside the mitochondria *via* the transmembrane potential of the inner membrane stimulated opening of the pore in rats at much lower concentrations than the neutral parent molecules; and they were also effective at inducing PTP opening in mouse and guinea pig mitochondria. This striking loss of species-specificity by cationic derivatives of NRB is consistent with our previous hypothesis that the neutral drug is taken up by mitochondria via a selective transport system that is restricted to the rat[1].

1. Ricchelli F., Dabbeni-Sala F., Petronilli V., Bernardi P., Hopkins B, & Bova, S. (2005) Species-specific modulation of the mitochondrial permeability transition by norbormide. *BBA-Bioenergetics* 1708: 178-186

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