Final report OTKA T049621

The involvement of mitochondrial-derived nitrogen radicals and mitoK_{ATP} channels in the regulation of organelle function.

Principal Investigator: Zsombor Lacza, MD, PhD
Department of Human Physiology and Clinical Experimental Research,
Semmelweis University, Budapest, Hungary
Total budget: 25 000 EUR
Period: 2004-2008

Abstract:

The goal of the present proposal was to identify the exact mechanisms of mitochondrial nitric oxide (NO) formation, ATP-dependent K-channel (mitoK_{ATP}) function, and their interactions and importance in oxidative stress related pathologies. first, we aimed to identify the subunit composition of mitoK_{ATP} channels in the human heart. Our experimental findings indicated that there previously suspected potential mitoK_{ATP} channel subunits are not present in the required format and amount in human heart mitochondria, therefore, they cannot play a role in K fluxes. This observation was in agreement with new results of other laboratories which forced us to abandon this line of research. However, the investigation of mitochondrial NO formation led to unexpected novel findings. First, we extended our preliminary results about the nature of the putative mitochondrial NO synthase (mtNOS) to several species and tissues (human, mouse, rat, pig, heart, brain and liver) and concluded that mtNOS is not a variant of any known NOS enzymes and that mitochondiral NO formation does not rely on the same enzymatic route. Our further efforts revealed that mitochondria are capable of producing significant amounts of nitrogen radicals through the catalyzation of nitrosothiol release by the ubiquinon cycle of the respiratory chain. The main thiol in the system is probably nitrosoglutathion (GSNO) which is capable of storing and releasing NO in the mitochondrial matrix. Next, we investigated the potential role of this mechanism in physiological and pathological processes. Subjecting mitochondria to oxidative stress resulted in the nitration and poly-ADPribosylation (PARylation) of proteins. Proteomic identification (2D gel electrophoresis and mass spectrometry) of the affected molecules revealed that dihydrolipoamide-dehydrogenase (DLDH), another respiratory chain enzyme is PARylated. Analyzing the substrates and catalytic function of the enzyme raised the possibility that it may act as a polyADP-ribose-polymerase (PARP), which was confirmed by various methodologies. Thus, we uncovered a completely new and independent mechanism of mitochondrial nitrosative stress, which is catalyzed by enzymes of the respiratory chain. Then, we concentrated our efforts on the potential role of mitochondrial oxidants in pathological reactions. Diabetic complications are related to endothelial dysfunction: the loss of NO production by diabetic vessels leads to microcirculatory disorders, while the increased cellular production of nitrogen radicals results in further tissue damage. We identified that unstable glucose levels result in prolonged oxidant production by mitochondria, which highlights that strict blood sugar control is more important than the average glucose level in the prevention of diabetic complications. In addition, we developed a GSNO containing gel which is capable of increasing local blood flow in both healthy and diabetic animals without any systemic circulatory or protein nitration effects.

During the 4 year duration of this grant we spent 25 000 EUR and published 10 peer-reviewed articles, 6 of which are first or last author papers and 3 are invited reviews with a total impact factor of 34. Unfortunately, the present level of funding is inadequate to continue research in a competitive field so we were forced to abandon our future plans in this line of research.

Összefoglaló

A pályázat célul tűzte ki a mitokondriális nitrogén monoxid (NO) termelés és K_{ATP} csatornák vizsgálatát, ezek egymásra hatását, illetve szerepét iszkémiás károsodásban. A munkaterv során elsőként a mitoKATP csatornák alegységeit terveztük azonosítani humán szív mintákon. A kísérletek során megbizonyosodtunk afelől, hogy a korábban ilyen csatornáknak gondolt fehérjék nincsenek jelen megfelelő számban és formában a mitokondriális membránokban, ezért a nem valószínű hogy a mitokondirális ATP-függő kálium áramokat valós mitoKATP csatornák folytatnák. Ez a megfigyelés egyezett több nemzetközi kutatócsoport eredményeivel, ezért a témának ezt a részét kénytelenek voltunk lezárni. A mitokondriális NO szintáz (mtNOS) enzimrendszer vizsgálata azonban nem várt új felfedezésekhez vezetett. Feltételezésünk szerint egy új, az eddig ismert NOS formáktól különböző enzim variáns található a mitokondriumokban. Az előkísérletekben is bemutatott, a pályázat során több fajra és szövetre (ember, egér, patkány, malac, szív, agy és máj) kiterjesztett, több párhuzamos módszerrel nyert eredményeink szerint bizonítottuk, hogy a feltételezett mtNOS nem azonos egyik ismert NOS variánssal sem, illetve nem ezek módosított változata. Sőt, megállapítottuk hgoy az NO termelés enzimatikus mechanizmusa sem lehet azonos a NOS kémiai működésével, ezért új reakció út létezését tételeztük fel. Kísérleteink során megállapítottuk, hogy a mitokondriális légzési lánc ubiquinon ciklusának működése mentén keletkező nitrogén gyökök azok, amelyeket korábban a mtNOS-nak tulajdonítottak. A rendszerben kulcsszerepet játszik a fehérjék, de különösen a tripeptid glutation nitrált változatai (nitrozótiolok, GSNO), amelyek NO szállítására, szabályozott leadására képesek. További kísérleteink ezen mechanizmus élettani szerepének, terápiás felhasználhatóságának tisztázására fókuszáltak. A reakcióút pontosabb tisztázása végett mitokondriumokat oxidatív stressznek vetettünk alá, ezzel fehérje modifikációt indukáltunk. Proteomikai módszerekkel (2D gélelektroforzis és tömegspektrometria) azonosítottuk nitrált és poly-ADP-rbozilált (PAR-ált) fehérjéket, köztük a mitokondirális dihidro-lipoamid dehidrogenázt (DLDH-t). Ez utóbbi enzim szerkezete, katalitikus folyamatának elemzése felvetette, hogy a sejtmagból már jól ismert patológiás reakció, a poliADP-riboziláció folyamatot is katalizálhatja, amelyet több módszerrel is igazolni tudtunk. Ezzel megállapítottuk, hogy a mitokondriumok légzési lánca először az ubiquinon ciklus által katalizált GSNO bomlás révén, majd az így keletkezett nitrogén gyökök által indukált, és ismét a légzési lánchoz tartozó DLDH segítségével PAR-ilációt okoznak. A kísérleteink ezzel egy teljesen új, független mitokondriális reakcióutat tártak fel. Ismert, hogy a NO-nitrozatív stressz-PARiláció útvonal jelentős szerepet játszik a cukorbetegség szövődményeinek, elsősorban a mikrovaszkuláris károsodásnak a kialakulásában. Megállapítottuk, hogy az ingadozó vércukorszint által kiváltott sejtszintű elváltozásokban jelentős szerepet játszik a mitokondriumok által később termelt oxidánsok jelenléte. Egy másik kísérletben azt vizsgáltuk, hogy a cukorbetegségben károsodott szöveti NO termelést pótolni lehet-e lokális GSNO adásával. Ez utóbbi kísérletek eredményeként új terápiás megoldást szabadalmaztattunk, amely a cukorbeteg láb szindróma kezelésére alkalmas lehet.

Az ifjúsági OTKA pályázatban 4 év alatt 7,4 millió forintos támogatás (2006-ban 10% elvonással) segítségével a munkaterv szerinti témában összesen 10 nemzetközi tudományos közlemény jelent meg, ebből 6 első vagy utolsó szerzős, 3 meghívott review, összesen 34 impakt faktorral. Mivel a szakterület műveléséhez szükséges minimális erőforrásokat hazai támogatásokból nem tudjuk biztosítani, ezért kénytelenek voltunk a nemzetközileg is kiemelkedő újdonságként szolgáló téma további kutatását a jelen pályázat lezártával megszüntetni.

Exploitation of results

During the course of our experiments with NO donors it became evident that mitochondria-derived NO donors may play a different role in cellular physiology than others. Thus, we investigated whether the main natural NO carrier molecule, which in present in mitochondria, can be beneficial in replacing the decreased NO production in diabetic complications. First, we identified that a GSNO containing hydrogel can dilate skin microvessels in diabetic animals. We also checked whether this gel gets absorbed in the systemic circulation and alters cardiac parameters or modifies protein nitration patterns at the site of application. Since neither suspected side effects occurred we concluded that a GSNO containing hydrogel is a new drug candidate for the treatment of diabetic microvascular disorders such as diabetic foot syndrome.
Our first goal was the development of a mitochondria-targeted GSNO product. However, the synthesis of a Schulachev-ion coupled GSNO was first achieved by the group of Brookes in the USA who patented this molecule. Their first experiments revealed that mitochondria targeted GSNO and native GSNO has the same pharmacological profiles, therefore the targeting in not essential. Unfortunately, GSNO is a very labile molecule with a half-life of only 5,5 hours in water solutions so we focused our efforts on creating a stabile hydrogel format which can be used as a clinical formulation.
Extensive experimentation with adjuvants and changing the physico-chemical environment of the hydrogel led to the discovers of two novel routes of stabilization:
1, Although the systhesis of GSNO is more effective at low pH, it is much more stabile in the 5-9 pH range.
2, Addition of polymers, especially the natural polymer chitosan decreases the spontaneous release of NO from the hydrogel.
We submitted patents for both mechanisms and we are actively seeking industrial partners for the development of this novel drug formulation.

Brief description of the results

Aim1: investigation of mitoK$_{ATP}$ channels

The mitochondrial ATP-sensitive K$^+$ (mitoK$_{ATP}$) channel was first described nearly fifteen years ago and it has been a major focus of several studies on neuroprotection, ischemic preconditioning and cardioprotection since then. Recently there is some controversy concerning the importance or even the existence of mitoK$_{ATP}$ channels. Although the structure of the ATP-sensitive K$^+$ channels of the plasma membrane is well characterized, the molecular composition of the mitoK$_{ATP}$ channel is still unclear. Previous experiments indicated that the known K$_{ATP}$ channel subunits – sulfonylurea receptor (SUR1, SUR2) and the inwardly rectifying potassium channel (Kir6.1, Kir6.2) are also present in mitochondria preparations. However, recent findings support the idea of a multiprotein complex in the mitochondria which has mitoK$_{ATP}$ channel activity and does not contain any of the putative mitoK$_{ATP}$ channel subunits. Despite its crucial role in ischemic preconditioning (IPC) and apoptosis, there are many questions about the molecular identity of the mitoK$_{ATP}$ remain unanswered. Therefore, we aimed to obtain a positive protein mass fingerprinting or de novo sequencing data for mitochondrial proteins, which share immunological characteristics of the known K$_{ATP}$ subunits such as SUR2 or Kir6.2. In order to reach this goal we separated mouse and human mitochondrial proteins by two dimensional gel electrophoresis followed by MALDI-TOF and Q-TOF analysis and amino acid sequencing. Our results show that both in cadaver and live human heart mitochondria and in mouse brain mitochondria the antibodies against the known K$_{ATP}$ channel subunits recognized subunits of the mitochondrial ATP synthase complex. Neither SUR nor Kir proteins were found. These results are in accordance with recent investigations from other laboratories, so we can conclude that a classical K$_{ATP}$ channel assembly cannot play a significant role in mitochondrial K$^+$ homeostasis.

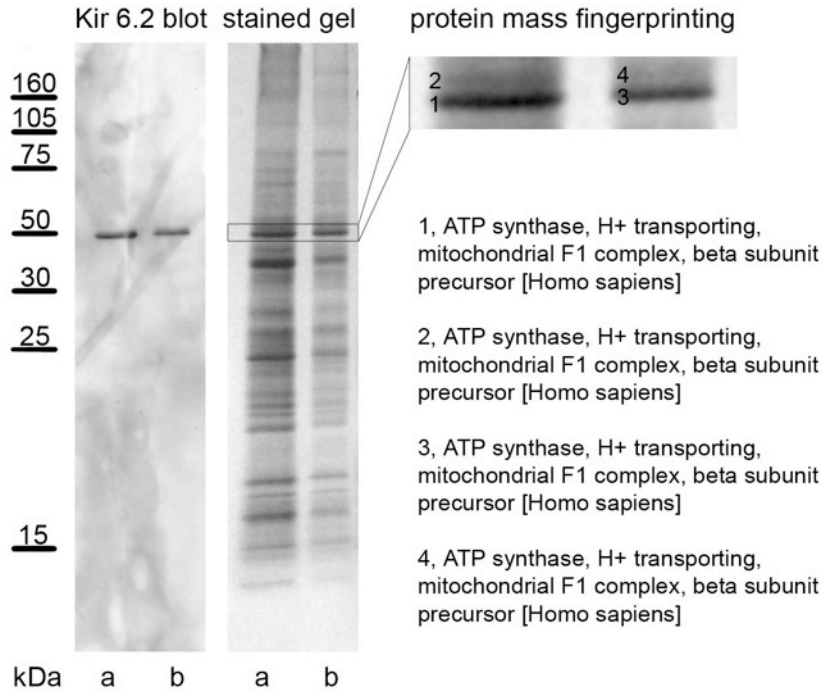

Fig. 1. Protein mass fingerprinting data of a putative mitoK$_{ATP}$ channel subunit Kir6.2.

Aim2: investigation of mitochondrial NO production

Previous studies raised the possibility that nitric oxide synthase is present in heart mitochondria (mtNOS) and the existence of such an enzyme became generally accepted. However, original experimental evidence is rather scarce and positive identification of the enzyme is lacking. We aimed to detect a NOS protein in human and mouse heart mitochondria and to measure the level of NO released from the organelles. Western blotting with 7 different anti-NOS antibodies failed to detect a NOS-like protein in mitochondria. Immunoprecipitation or substrate-affinity purification of the samples concentrated NOS in control preparations but not in mitochondria.

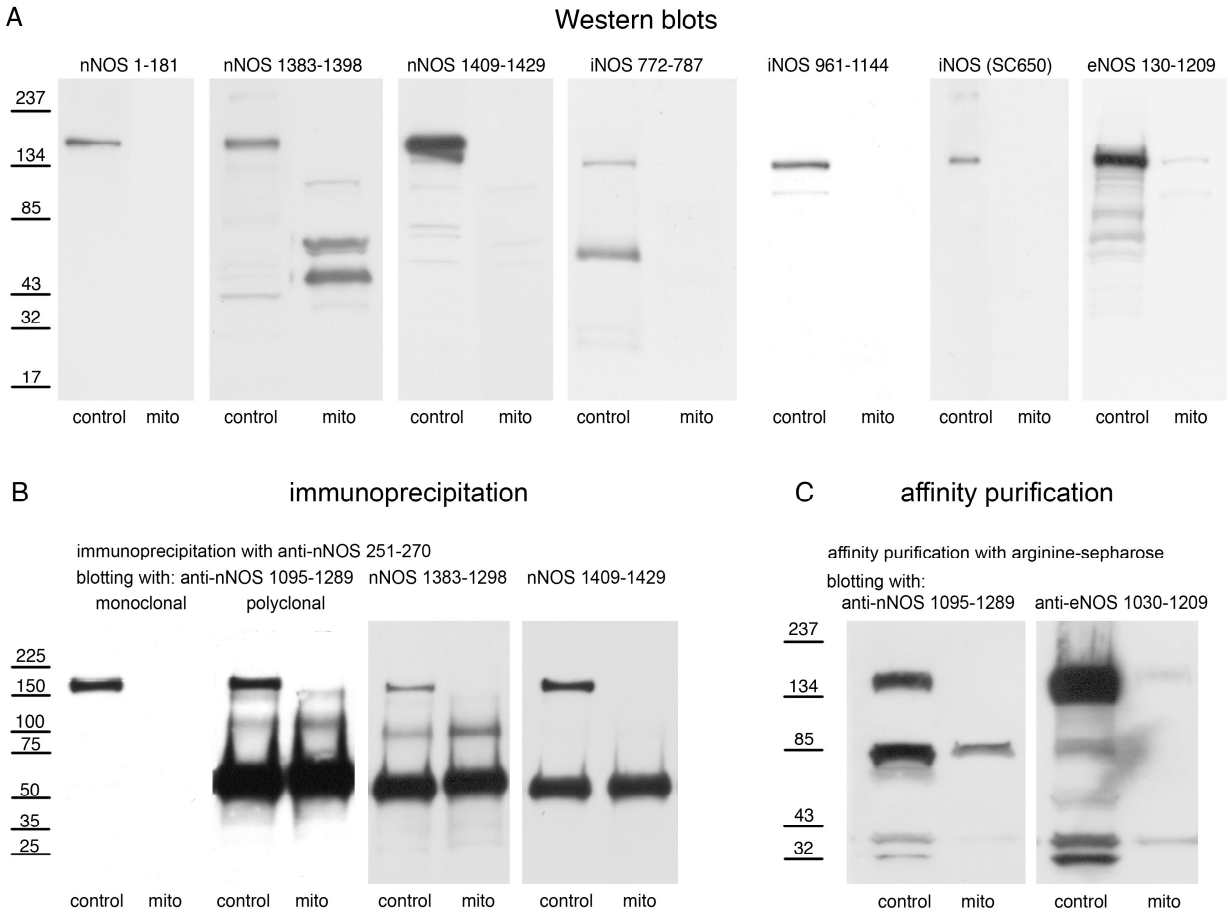

Fig. 2. Search for NOS isoforms in mouse heart mitochondria. Panel A shows representative Western blots against the known NOS isoforms in mitochondria and positive control tissues (brain, activated macrophage, heart for nNOS, iNOS or eNOS, respectively). The epitopes of the corresponding antibodies are shown by their respective locations in the protein sequences. Panel B shows immunoprecipitation with an anti-nNOS antibody, followed by Western blotting with other nNOS antibodies against different epitopes. Note the strong nNOS band in the controls and the lack of it in the mitochondria regardless of the antibody used. Panel C shows concentration of arginine-binding proteins with arginie-sepharose followed by Western blotting.

Release of NO from live respiring human mitochondria was below 2 ppb after 45 min of incubation. In a bioassay system, mitochondrial suspension failed to cause vasodilation of human mammary artery segments. These results indicate that mitochondria do not produce physiologically relevant quantities of NO in the heart and are unlikely to have any physiological importance as NO donors in the heart, nor they contain a genuine mtNOS enzyme. Therefore, if mtNOS cannot be responsible for mitochondrial NO production, we investigated other possible mechanisms. We measured the

contribution of respiratory chain enzymes to reactive nitrogen species (RNS) production. In a separate methodological study we investigated the reaction of the fluorescent dyes DAF and DAR with various nitrogen and oxygen radicals and concluded that both dyes react primarily with N_2O, a reaction product of NO and O_2. Diaminofluorescein (DAF) was applied for the assessment of RNS production in isolated mouse brain, heart, and liver mitochondria and also in a cultured neuroblastoma cell line by confocal microscopy and flow cytometry. Mitochondria produced reactive nitrogen species, which was inhibited by catalysts of peroxynitrite decomposition but not by nitric oxide synthase inhibitors. Disrupting the organelles or withdrawing respiratory substrates markedly reduced RNS production. Inhibition of Complex I abolished the DAF signal, which was restored by Complex II substrates. Inhibition of the respiratory complexes downstream from the ubiquinone/ubiquinol cycle or dissipating the proton gradient had no effect on DAF fluorescence. We conclude that mitochondria from brain, heart and liver are capable of significant RNS production via the respiratory chain rather than through an arginine-dependent mitochondrial nitric oxide synthase.

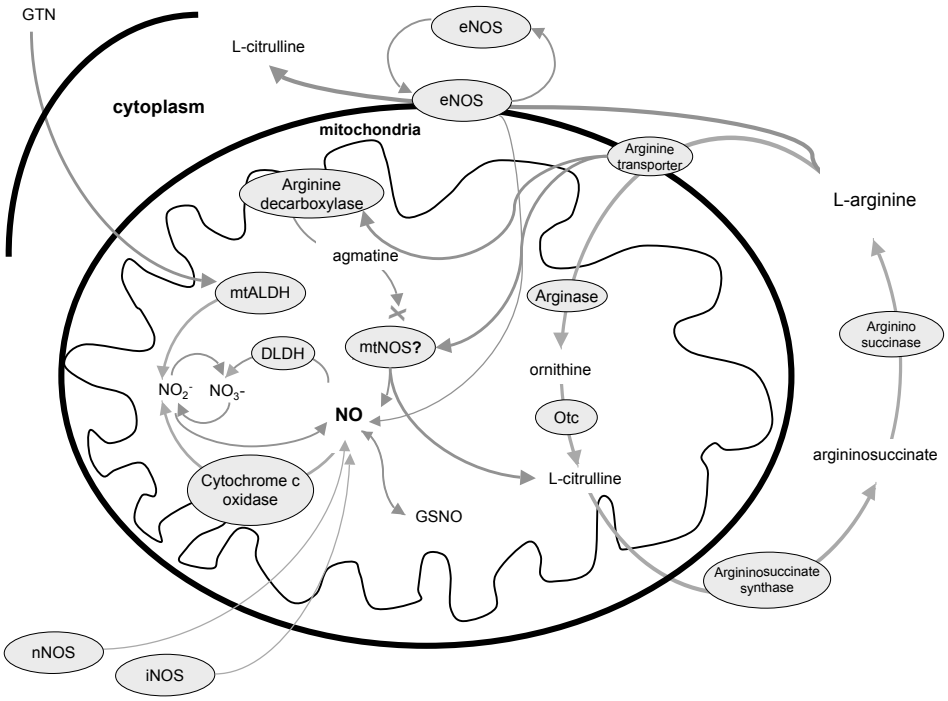

Fig. 3. Potential sources and metabolism of NO and related compounds in mitochondria. Arrows indicate chemical reactions, a cross indicates blocking effect. Abbreviations: DLDH: dihydrolipoamide-dehydrogenase, eNOS: endothelial nitric oxide synthase, GTN: glyceryl-trinitrate, iNOS: inducible nitric oxide synthase, GSNO: nitrosoglutathione, mtALDH: mitochondrial aldehyde dehydrogenase, mtNOS: mitochondrial nitric oxide synthase, nNOS: neuronal nitric oxide synthase, NO: nitric oxide, Oct: ornithine-citrulline transaminase.

The significant levels of mitochondrial RNS production raised the question whether other elements the cellular poly-ADP ribosylation cascade is also present in mtichondria. In the next series of experiments we found several poly-ADP-ribosylated proteins in isolated rat liver mitochondria following hydrogen peroxide (H2O2) or nitric oxide donor treatment. Protein poly-ADP-ribosylation was more intense in isolated mitochondria than in whole tissue homogenates and it was not associated with increased nuclear PARP activity.

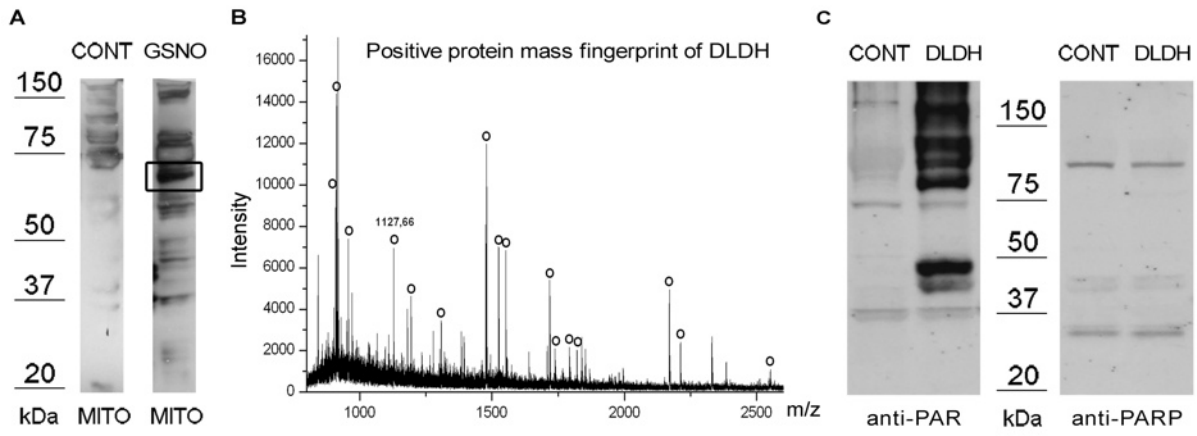

Fig. 4. DLDH contributes to mitochondrial PARP-like enyzme activity. Treating live mitochondria with 3 mM GSNO resulted in extra PAR bands (panel A). One of the PARylated proteins was identified by mass spectrometry as dihydrolipoamide dehydrogenase (panel B). Incubation of mitochondria with recombinant DLDH enzyme caused intense PARylation, but that was not due to the activation of the PARP enzyme (panel C).

We identified five poly-ADPribose (PAR) positive mitochondrial bands by protein mass fingerprinting. All of the identified enzymes exhibited decreased activity or decreased levels following oxidative or nitrosative stress. One of the identified proteins is dihydrolipoamide dehydrogenase (DLDH), a component of the alpha-ketoglutarate dehydrogenase (KGDH) complex, which uses NAD+ as a substrate. This raised the possibility that KGDH may have a PARP-like enzymatic activity. The intrinsic PARP activity of KGDH and DLDH was confirmed using a colorimetric PARP assay kit and by the incubation of the pure enzymes with H2O2 and the nitric oxide donor S-nitrosoglutathione (GSNO). The KGDH enzyme may, therefore, have a novel function as a PARP-like enzyme, which may play a role in regulating intramitochondrial NAD+ and poly(ADP-ribose) homeostasis, with possible roles in physiology and pathophysiology.

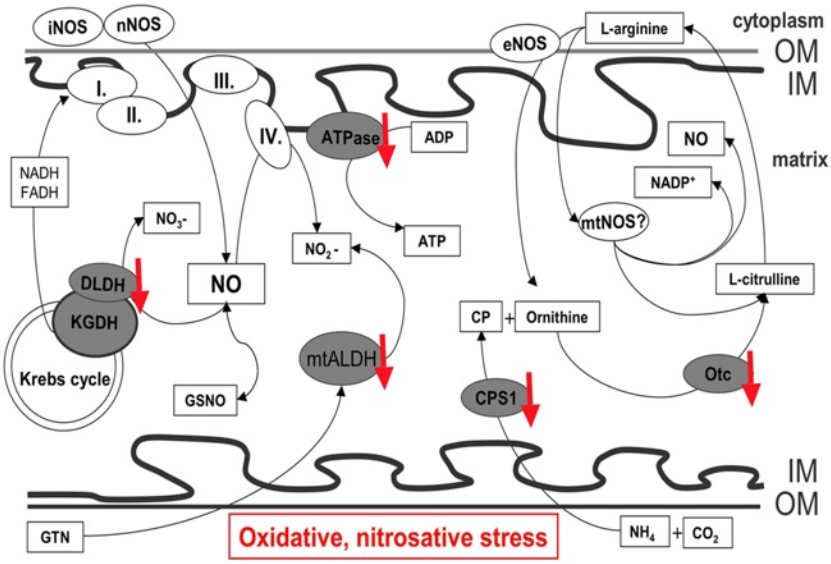

Fig. 5. Summary of mitochondrial NO, nitrogen radical, and PAR metabolism.

Publications supported from this grant:

1, E. Pankotai, Z. Lacza, M. Murányi, C. Szabó (2009) Intra-mitochondrial poly(ADP-ribosyl)ation: potential role for alphaketoglutarate dehydrogenase. Mitochondrion, in press, IF: 3,209

2, Z. Lacza, E. Pankotai, D.W. Busija: Mitochondrial nitric oxide synthase: current concepts and controversies. (2009) Frontiers in Bioscience, E1:218-225, IF: 3.226

3, M. A. Ihnat, J. E. Thorpe, C. D. Kamat, C. Szabó, D. E. Green, L. A. Warnke, Z. Lacza, A. Cselenyák, K. Ross, S. Shakir, L. Piconi, R. C. Kaltreider, A. Ceriello: Reactive oxygen species mediate a cellular 'memory' of high glucose stress signaling. (2007) Diabetologia 50(7):1523-31. IF: 5,583

4, A. Csordás, E. Pankotai, J. A. Snipes, A. Cselenyak, Z. Sárszegi, A. Cziráki, B. Gaszner, L. Papp, R. Benkő, L. Kiss, E. Kovács, M. Kollai, C. Szabó, D. W. Busija, Z. Lacza (2007) Human heart mitochondria do not produce physiologically relevant quantities of nitric oxide. Life Sciences 80:633-7, IF: 2.512,

5, A.B. Seabra, E. Pankotai, M. Fehér, Á. Somlai, L. Kiss, L. Bíró, C. Szabó, M. Kollai, M. G. de Oliveria, Z. Lacza (2007) S-nitrosoglutathione-containing hydrogel increases dermal blood flow in streptozotocin-induced diabetic rats. British Journal of dermatology, 156(5):814-8, IF: 2.978

6, Molnar A, Toth A, Bagi Z, Papp Z, Edes I, Vaszily M, Galajda Z, Papp JG, Varro A, Szuts V, Lacza Z, Gero D, Szabo C. (2006) Activation of the poly(ADP-ribose) polymerase pathway in human heart failure. Molecular Medicine 12(7-8): 143-52. IF: 3,190

7, Z Lacza, AV Kozlov, E Pankotai, A Csordas, G Wolf, H Redl, M Kollai, C Szabo, DW Busija, TF Horn: Mitochondria produce reactive nitrogen species via an arginine-independent pathway. (2006) Free Radic Res. 40(4):369-78 IF: 2.744

8, Z Lacza, E Pankotai, A Csordás, D. Gerő, L. Kiss, E. M. Horváth, M Kollai, D. W. Busija, C. Szabó: Mitochondrial NO and reactive nitrogen species production: does mtNOS exist? (2005) Nitric Oxide - Biology and Chemistry 14(2): 162-168 IF: 3,314

9, Z Lacza, E. M. Horváth, E Pankotai, A Csordás, M Kollai, C. Szabó, D. W. Busija: The novel red-fluorescent probe DAR-4M measures reactive nitrogen species rather than NO. (2005) Journal of Pharmacological and Toxicological Methods 52(3): 335-340 IF: 0,625

10, D. W. Busija, Z. Lacza, N. Rajapakse, K. Shimizu, B. Kis, F. Bari, F. Domoki and T. Horiguchi: Targeting mitochondrial ATP-sensitive potassium channels—a novel approach to neuroprotection. Brain Research Reviews (2005) 46: 282-294, IF: 6.504