THE EFFECT OF CALCIUM ON REACTIVE OXYGEN SPECIES GENERATION IN ISOLATED MITOCHONDRIA

DOCTORAL THESES

Zsófia Komáry M.D.

SEMMELWEIS UNIVERSITY
SZENTÁGOTHAI JÁNOS PHD SCHOOL OF NEUROLOGICAL SCIENCES

Supervisors:	Vera Adam-Vizi M.D., regular member of the Hungarian Academy of Sciences, professor László Tretter M.D., D.Sc., professor
Opponents:	Balázs Sümegi M.D., D.Sc., professor Tamás Kardon M.D., PhD, adjunct professor
Head of the exam committe:	József Mandl M.D., regular member of the Hungarian Academy of Sciences, professor
Members of the exam committee:	Tibor Zelles M.D., PhD Ferenc Gallyas M.D., PhD, professor

Budapest 2012

LIST OF PUBLICATIONS

Komary Z, Tretter L, Adam-Vizi V (2008) H_2O_2 generation is decreased by calcium in isolated brain mitochondria. *Biochimica et Biophysica Acta* **1777:** 800-807 (IF: 4.447)

Komary Z, Tretter L, Adam-Vizi V (2010) Membrane potential-related effect of calcium on reactive oxygen species generation in isolated brain mitochondria. *Biochimica et Biophysica Acta* **1797:** 922-928 (IF: 5.132)

THE EFFECT OF CALCIUM ON MITOCHONDRIAL ROS PRODUCTION IN CASE OF mPTP OPENING

In the absence of adenine nucleotides calcium induced mPTP opening. In our experiments calcium induced opening of mPTP in the presence of both complex I and complex II respiratory substrates, decreased mitochondrial ROS production.

CONCLUSION

Our results do not support the commonly accepted hypothesis that ROS production of mitochondria accumulating calcium in pathological conditions is markedly increased. With our work we rather would like to emphasize that the effect of calcium on ROS production depends mainly on the metabolic state of mitochondria and on the magnitude of the calcium insult.

INTRODUCTION

Glutamate excitotoxicity is a key element in the pathophysiology of acute and chronic neurological disorders.

During glutamate excitotoxicity long term activation of glutamate receptors of the central nervous system results in neuronal damage. In the pathophysiology of neuronal death elevation of intracellular calcium concentration and increased cellular reactive oxygen species (ROS) generation are significant phenomena. The enhanced cellular ROS production can originate from various sources. Calcium can activate phospholipase A_2 enzyme which releases arachidonic acid, the latter produces superoxide anion during its further metabolism. Calcium at the same time can activate nitric oxide synthase, and superoxide anion producing xanthine oxidase and NADPH oxidase enzymes. During glutamate excitotoxicity mitochondria might also contribute to enhanced cellular ROS generation. The role of

mitochondria are supported by studies on neuronal cell culture in which accelerated cellular ROS production after glutamate exposure can be modulated by mitochondrial respiratory complex inhibitors and uncouplers.

In our experiments we examined the effect of calcium on ROS production in isolated brain mitochondria.

The ROS generation of isolated mitochondria and the effect of calcium on ROS production are highly influenced by adenine nucleotides, inhibitors of respiratory complexes, the type of respiratory substrates, mitochondrial $NADH^{+}+H^{+}$/ NAD^{+} ratio and opening of the mitochondrial permeability transition pore (mPTP). Varying applied experimental conditions can explain the diversity of the literature on the effect of calcium on ROS production in isolated mitochondria: ROS production increasing and decreasing effect of calcium are both supported by literature data.

In our work our aim was to clarify the effect of calcium on mitochondrial ROS production, and to define the

inner membrane, therefore reduced mitochondrial ROS generation.

3.) In the presence of succinate as repiratory substrate, at high $\Delta\Psi m$ reverse electron transport takes place. *Via* RET a portion of electrons moves backwards to complex I and reduce NAD^{+}, at the same time superoxide anion is produced on complex I. RET and the high ROS production of RET is tightly membrane potential dependent, therefore calcium reduced ROS production of RET due to its depolarizing effect.

4.) With succinate, at low membrane potential induced by ADP RET already does not work, the calcium induced further depolarization did not alter mitochondrial ROS production, since under the membrane potential value created by ADP mitochondrial ROS production is not influenced by $\Delta\Psi m$.

the low membrane potential value induced by ADP. This calcium concentration increased the ADP induced low ROS production nearly twofold, because calcium elevated ΔΨm to a range in which mitochondrial ROS production depends on ΔΨm. The calcium induced elevation of mitochondrial $NADH+H^+/ NAD^+$ ratio could also contribute to the enhanced mitochondrial ROS production.

In phosphorylating mode the effect of calcium on ROS generation depends on the magnitude of the calcium insult as well. In our experiments 300 μM [Ca^{2+}] caused sustained depolarization: ΔΨm decreased under the low membrane potential value induced by ADP. In this low membrane potential range mitochondrial ROS production is not ΔΨm-dependent, therefore 300 μM [Ca^{2+}] did not alter mitochondrial ROS production.

2.) In mitochondria respiring on glutamate plus malate, at high membrane potential values created by ATP or ADP plus oligomycin, calcium depolarized the mitochondrial

mechanisms according to which calcium modulates mitochondrial ROS generation. With our results we wish to contribute to the interpretation of diverse literature data.

PURPOSES

With our experiments we wanted to answer the following questions:

1. Does calcium influence ROS production of mitochondria?

1.1. How does mitochondrial membrane potential ($\Delta\Psi_m$) alter the effect of calcium on mitochondrial ROS production?

1.2. Does the type of respiratory substrates modulate the effect of calcium on mitochondrial ROS production?

1.3. How does calcium induced mPTP opening influence the mitochondrial ROS production?

1.4. How does the amount and the redox state of pyridine nucleotides change upon the effetct of calcium and upon mPTP opening induced by calcium?

METHODS

PREPARATION OF ISOLATED MITOCHONDRIA

Mitochondria were isolated from guinea pig brain cortex via differential centrifugation using Percoll gradient. Before measurements respiratory controll ratio of isolated mitochondria was defined by Clark-type oxygen electrode.

DETERMINATION OF CALCIUM CONCENTRATION

The added total calcium concentrations were determined by Chelator software. In the assay medium we measured the free calcium concentration with Fura-6F fluorescent dye.

RESULTS

Based on our results we can conclude that calcium neither has a specific mitochondrial target, nor a definite mechanism which would uniformly determine the effect of calcium on mitochondrial ROS production. Instead, calcium performs its effect indirectly, by modulating i) mitochondrial membrane potential, ii) ROS producing mechanism of succinate *i.e.* reverse electron transport (RET), iii) mitochondrial $NADH+H^+$/ NAD^+ ratio and iv) opening of mPTP.

ΔΨm-DEPENDENT EFFECT OF CALCIUM ON MITOCHONDRIAL ROS PRODUCTION

1.) In mitochondria respiring on glutamate plus malate, at low ΔΨm induced by ADP, 50 μM [Ca^{2+}] after a transient depolarization hyperpolarized mitochondrial inner membrane, which means that calcium elevated ΔΨm above

DETERMINATION mPTP WITH TRANSMISSION ELECTRON MICROSCOPE

Isolated mitochondria were centrifuged and pellet was fixed in glutaraldehyde and sodium cacodylate, postfixed with osmium tetroxide, dehydrated with alcohol and propylene oxide, finally mitochondria were embedded in Durcupan. Sections were observed with JEOL 1200 EMX transmission electron microscope.

MITOCHONDRIAL CALCIUM UPTAKE

In case of 50 μM [Ca^{2+}], calcium uptake was measured with calcium green-5N, in case of 300 μM [Ca^{2+}], calcium uptake was detected with Rhod-5N fluorescent dye. Fluorescence was measured with PTI Deltascan fluorescence spectrophotometer. Fluorescence intensity was calibrated with a calibration scale formed with pulses of known amount of calcium.

MEASUREMENT OF MITOCHONDRIAL H_2O_2 PRODUCTION

Mitochondrial H_2O_2 production was determined extramitochondrially with Amplex Red fluorescent dye. Amplex Red in the presensce of horseradish peroxydase reacts with H_2O_2 with the stoichiometry: 1:1, resulting in fluorescent resorufine. We initiated H_2O_2 generation with glutamate and malate or succinate respiratory substrates. We measured fluorescence with Photon Technology International (PTI) Deltascan fluorescence spectrophotometer. At the end of each measurement we performed calibration with known amount of H_2O_2.

MEASUREMENT OF NAD(P)H+H^+ AUTOFLUORESCENCE

We measured mitochondrial NAD(P)H+H^+ autofluorescence in parallel with H_2O_2 detection with PTI Deltascan fluorescence spectrophotometer.

MEASUREMENT OF MITOCHONDRIAL NAD^++ $NADH+H^+$ POOL

For determining mitochondrial NAD^++ $NADH+H^+$ pool we permeabilized mitochondrial membranes with Triton X-100 detergent and we put mitochondria in an assay medium containing alcohol dehydrogenase, 3-(4,5-dimethylthiazole-2-yl)-2.5-diphenyl tetrazolium bromide (MTT), phenazine ethosulphate (PES) and ethanol. We measured the absorbance of MTT with GBC UV/VIS 920 spectrophotometer. For calibration we used known amount of NAD^+.

DETERMINATION OF $\Delta\Psi_m$

We determined $\Delta\Psi_m$ with Safranine O cationic fluorescent dye which accumulates in the matrix due to its positive charge. We determined $\Delta\Psi_m$ with tetramethyl-rhodamine methyl esther (TMRM) as well. We detected fluorescence with PTI Deltascan fluorescence spectrophotomemer or with Hitachi F-450 spectrofluorimeter. We did not calibrate

fluorescence signal, therefore we could evaluate the results only qualitatively.

DETERMINATION OF MITOCHONDRIAL SWELLING

We determined swelling of mitochondria with light scattering with PTI Deltascan fluorescence spectrophotometer or with Hitachi F-450 spectrofluorimeter. We measured swelling in parallel with $\Delta\Psi_m$ measurement.

DETERMINATION OF mPTP WITH CALCEIN FLUORESCENCE

We incubated mitochondria in a buffer containing the acetoxymethyl esther form of calcein (calcein-AM). From the membrane permeable calcein-AM mitochondrial estherases release free fluorescent calcein, which is captured within mitochondria because of its hydrophilic character. The assay medium contained $CoCl_2$ which quenches the fluorescence of calcein in case of mPTP opening. We detected fluorescence with PTI Deltascan fluorescence spectrophotometer.