SHORT THESIS FOR THE DEGREE OF DOCTORAL OF PHILOSOPHY (PHD)

The role of PARP-2 in the regulation of autophagy and mitochondrial morphology

by Laura Jankó

Supervisor: Dr. Péter Bay

UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF MOLECULAR MEDICINE

DEBRECEN

The role of PARP-2 in the regulation of autophagy and mitochondrial morphology

By Laura Jankó, Molecular Biology MSc

Supervisor: Péter Bay, PhD, DSc

Doctoral School of Molecular Medicine, University of Debrecen

Head of the Defense Committee:	László Csernoch, PhD, DSc
Reviewers:	András Mádi, PhD Tibor Pankotai, PhD
Members of the Defense Committee:	András Balla, PhD Endre Károly Kristóf, PhD

The PhD Defense (online format) takes place at 1:00 pm, 25th of January, 2022. Publicity is guaranteed during the online Defense. If you are willing to participate, please indicate via e-mail to janko.laura@med.unideb.hu until 4:00 pm on 24th of January, 2022. Due to technical reasons later sign-ups are not possible and you will not be able to join the online Defense.

Introduction

Members of the PARP family share a domain with catalytic activity that cleaves NAD^+ to nicotinamide and ADP-ribose and synthesizes large PAR polymers using ADP-ribose units. The PARylation reaction catalyzed by PARP enzymes is an evolutionarily conserved posttranslational modification of proteins. PARP-2 is a member of this protein family and PARP-2 is responsible for 10-15% of intracellular PARP activity. PARP-2 was first described in DNA repair, but its role has since been described in a number of physiological and pathophysiological processes such as regulation of gene expression and mitochondrial processes, lipid metabolism, cellular response to oxidative damage, spermatogenesis, T-cell maturation and immune processes.

Relationship between PARP enzymes and autophagy

In order to maintain the homeostasis of eukaryotic cells, a balance of anabolic and catabolic processes is essential. Autophagy is an evolutionarily conserved intracellular self-digestive process in which a cell degrades its own macromolecules or organelles using the lysosomal degradation system. Moreover, autophagy plays an important role in protection against pathogens. After degradation by lysosomal hydrolases, monomers are transported to the cytosol by their respective transporters. The main regulators of autophagy are the cellular energy level sensors, mTOR complexes and AMPK. At normal nutrient supply, mTORC1 inhibits the initial steps of autophagy, whereas nutrient depletion induces autophagy. AMPK is able to regulate autophagy in both mTORC1-dependent and -independent ways. In ATP depletion, AMPK inhibits mTORC1 activity and induces autophagy. However, increased concentration of ATP inhibits AMPK activity. Autophagy is a tightly regulated process, under- or over-functioning of its optimal level can lead to cell death. The dysregulation of autophagy has been demonstrated in a number of pathophysiological processes, such as neurodegenerative diseases and tumorigenesis.

Two members of the PARP family, PARP-1 and PARP-10, can involve in the regulation of autophagy. PARP-1 was identified as a proautophagic factor that induces autophagy in cases of severe cytostatic damage. PARP-10 was described as an interaction

partner of p62, which has a role in autophagosomal protein degradation, but the role of PARP-10 is unclear in the regulation of autophagy.

The role of PARP-2 in mitochondrial biogenesis

Mitochondria are organelles that can be called as the energy-producing centers of eukaryotic cells. Mitochondria can show different shapes, sizes and quantities depending on the cell types and the differentiation state of cells. One of the main tasks of the mitochondria is the ATP synthesis during oxidative phosphorylation, so it can conventionally be called the energy-producing center of cells. However, mitochondria play a role not only in ATP synthesis but also in many other intracellular processes, such as intracellular calcium concentration or the initial steps of apoptosis. Many essential biosynthetic pathways are localized in the mitochondrial matrix, so the flawless function of mitochondria is also essential for the efficient function of eukaryotic cells. Mitochondrial quality control consists of well-regulated processes. Negative changes in mitochondrial dynamics, mitophagy, mitochondrial ROS production, and mtUPR may lead to pathophysiological processes such as cardiomyopathy, diabetes mellitus, neurodegeneration, or arteriosclerosis.

The relationship between PARP-2 and mitochondria has been identified in several cases. PARP-2 depletion results in SIRT1 activation, which causes mitochondrial biogenesis and increases the oxidative metabolism by increasing the expression of major mitochondrial enzymes. In addition, enhanced mitochondrial content and mtDNA levels were identified in PARP-2 knockout mice, which correlated with the oxidative phenotype observed in muscle and liver in the absence of PARP-2.

Aims

We would like to expand our knowledge about the role of PARP-2 in metabolism in the direction of one of the most important intracellular degrading process, autophagy. In order to explore the relationship, we set the following goals in our work:

1. Investigation of the changes in autophagy during PARP-2 deficiency.
2. Examination of the activity change of energy sensors involved in the regulation of autophagy upon the silencing of PARP-2.
3. Exploration of the effects of increased SIRT1 activation on autophagy in the absence of PARP-2.

PARP-2 plays a role in the regulation of mitochondrial homeostasis. We aim to broaden our knowledge in this regard. Accordingly, our main objectives were the following:

1. Investigation of mitochondrial morphological changes induced by PARP-2 deficiency.
2. Study of oxidative stress processes resulting from silencing of PARP-2.
3. Examination of the mitochondrial effects of PARP-1 and PARP-3.

Material and methods

Chemicals

GSH, chloroquine, MitoTEMPO, NAC, PJ34 and resveratrol were from Sigma-Aldrich. EX-527 and olaparib were obtained from Selleckchem. AICAR was purchased from Santa Cruz Biotechnology. NR was from ChromaDex and rapamycin was from Cayman Chemical.

Cell Culture

C2C12 myoblasts and HepG2 hepatocarcinoma cells were maintained in DMEM containing 10% fetal bovine serum, 1% penicillin/streptomycin, and 2 mM L-glutamine at 37°C with 5% CO_2. PARP-2 was silenced by specific shRNA in both cell lines and maintained over extended periods by selection with puromycin. Cells that harbor the plasmid expressing the small hairpin RNA against PARP-2 are referred to as shPARP2, while cells that harbor the plasmid expressing a small hairpin RNA that has no target in human or murine cells (controls) are referred to as scPARP-2.

MEF cells were generated from PARP-2 knockout (PARP-$2^{-/-}$) and wild-type (PARP-$2^{+/+}$) mice for a previous study. Cells were cultured in DMEM containing 20% FBS, 1% penicillin/streptomycin, and 2 mM L-glutamine at 37°C with 5% CO_2.

Transient transfection

Transient transfection was performed in C2C12 cells for PARP-2, and in scPARP2 and shPARP2 C2C12 cells for SIRT1, PARP-1, and PARP-3 genes. Silencing of PARP-2, PARP-1, and PARP-3 genes we used siRNAs with 3 different sequences, designated # 1, # 2, and # 3, respectively. Untransfected, control cells and cells transfected with negative control siRNA were used in all experiments.

Cells were transfected with siRNA at final concentration of 30 nM using Lipofectamine™ RNAiMax reagent. Cells were assayed 48 hours post-transfection. The effectiveness of silencing was verified by RT-qPCR and Western blotting before experiments.

***In vitro* cell proliferation assay**

Sulphoradamine B (SRB) assay was applied to detect cell proliferation that measures changes in total protein content. Cells were fixed by 50% TCA for 1 hour, then cells were stained with 0.4% sulforhodamine B solution for 10 minutes. Unbound dye was removed by washing steps with 1% acetic acid, and then bound stain was solubilized with 10 mM Tris base. Absorbance was read on plate reader at 540 nm.

Detection of Cell Death

To evaluate changes in apoptotic and necrotic cell death, a FITC Annexin V/Dead Cell Apoptosis Kit was used. Cells were collected and stained with 5 µL FITC Annexin V and 100 µg/mL of PI for 15 minutes. Cells were measured by FACSCalibur™ flow cytometry, and data were analyzed using BD CellQuest Pro™ software.

Confocal microscopy

We used a Leica TCS SP8 confocal microscope with LAS X software for the analysis of our samples. Processed images were analyzed using ImageJ software. Mito-Morphology Macro was used for the morphological analysis of mitochondria, the mitochondrial content, perimeter, circularity, and form factor were analyzed. Form factor is derived from the area-to-perimeter ratio; hence its decrease would signify fragmentation. For co-localization analysis and the assessment of the Pearson correlation coefficient, ImageJ software with EzColocalization plug-in was used.

Study of the mitochondrial network

For the examination of mitochondria, we used MitoTracker ™ Red CMXRos fluorescent dye, which is able to accumulate in the mitochondria. Cells grown on glass coverslips, then cells were stained with MitoTracker™ Red CMXRos at final concentration of 100 nM for 20 minutes. Cells were washed with PBS and fixed with 4% paraformaldehyde for 10 minutes.

Investigation of acidic organelles

To study acidic vesicles (lysosomes, autolysosomes) in the cells, we used LysoTracker™ Deep Red fluorescent dye, which accumulates protonated form after entering the organelles. Cells grown on glass coverslips, then cells were stained with LysoTracker™ Deep Red at final concentration of 50 nM for 30 minutes. After staining, cells were washed with PBS and fixed with 4% paraformaldehyde for 10 minutes.

Immunofluorescence

Cells grown on glass coverslips, then fixed with 4% paraformaldehyde and permeabilized with 1% Triton X-100 in PBS. Between each steps, cells were rinsed three times with PBS. Cells were blocked with 1% BSA in PBS for 1 hour, then cells were incubated with primary antibodies overnight. Next day, fluorophore-labeled secondary antibody was added to the samples and nuclei were visualized with DAPI.

Total RNA Preparation and RT-qPCR

Total RNA was prepared using TRIzol reagent according to the manufacturer's instructions. The purity and quantity of RNA samples were measured with NanoDrop™ 1000 spectrophotometer.

We used 2 µg of total RNA for reverse transcription reaction using a High-Capacity cDNA Reverse Transcription Kit.

cDNA samples were diluted 10-fold and used in qPCR reactions using qPCRBIO SyGreen Mix Lo-ROX. Measurements were performed with a LightCycler 480 Real-Time PCR instrument. Expression was normalized to the geometric mean of murine 36B4 and cyclophilin values.

Determination of cellular ATP level

Cellular ATP level was determined using an ATP Assay Kit. Cells were collected and lysed in ATP Assay Buffer. Fluorescence was measured in 96-well black plates using a Spark 20M fluorometer. Based on the measured fluorescence, ATP concentration was determined. ATP levels were normalized to protein content, and normalized readings are presented.

SDS-PAGE and Western blotting

Cells were washed with PBS, collected and lysed in lysis buffer (50 mM Tris, pH 8, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF, protease inhibitor cocktail) on ice. The protein content was determined using the Pierce™ BCA Protein Assay Kit and boiled with 5xSDS sample buffer (310 mM Tris-HCl, pH 6.8, 50% glycerol, 10% SDS, 100 mM DTT, 0.01% bromophenol blue) and 2-mercaptoethanol for 10 minutes at 100°C.

Protein extracts were separated by SDS polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were blocked with 5% BSA in TBS_{Tween} for 1 hour at room temperature and incubated with primary antibodies overnight. Membranes were washed with TBS_{Tween} and probed with the respective peroxidase-conjugated secondary antibody. Signals were visualized by enhanced chemiluminescence reaction and captured by ChemiDoc™ Touch Imaging System. Bands were quantified by densitometry using ImageJ software and densitometry data were analyzed by statistical methods.

Measurement of superoxide production

Superoxide production was measured using dihydroethidium (DHE) staining, that after oxidation in the cytosol, able to intercalate into nuclear DNA. Cells were collected, then stained with 2.5 µM DHE for 30 minutes. Fluorescence was analyzed by FACSCalibur™ flow cytometry. For data evaluation, BD CellQuest Pro™ software was used.

Determination of lipid peroxidation

Lipid peroxidation was assessed by determining the production rate of thiobarbituric acid-reactive substances and by Western blotting of 4HNE-modified proteins. Cells were washed with PBS and collected. A solution of 8.1% SDS, 20% acetic acid, 0.8% thiobarbituric acid and distilled water was added to the cells and heated at 95°C for 1 hour. Samples were cooled down, centrifuged, and the absorbance of the supernatant was measured with a spectrophotometer at 530 nm. The lipid peroxidation was normalized to the cell number and these normalized values were plotted.

Determination of the oxygen consumption rate

The oxygen consumption rate was determined using a Seahorse XF96 Flux Analyzer. Cells were seeded into 96-well assay plates. After recording the baseline, the OCR was recorded every 5 minutes, and 50 µM etomoxir was used for determining the fatty acid oxidation. Data were normalized to protein content, and normalized readings are displayed.

Electron microscopy

We examined the ultrastructure of scPARP2 and shPARP2 C2C12 and scPARP2 and shPARP2 HepG2 cells by electron microscope.

Cells were collected, then cells were fixed in 3% glutaraldehyde dissolved in 0.1 M cacodylate buffer (pH 7.4) containing 5% sucrose. After washing in 0.1 M cacodylate buffer (pH 7.4), cells were post-fixed in 1% osmium tetroxide dissolved in 0.1 M cacodylate buffer (pH 7.4). Then, cells were dehydrated with a graded ethanol series. Samples were embedded into Durcupan™ ACM resin. Ultrathin sections were cut with Leica Ultracut UCT Ultramicrotome, collected on Formvar-coated single-slot grids, and counterstained using uranyl acetate and Reynolds lead citrate. Sections were examined with a JEOL 1010 transmission electron microscope.

Morphometric assessment was accomplished using the ImageJ software. EM pictures of at least 45 different cells of each group were analyzed.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software. All groups were checked for normal distribution. For comparing two groups, two-sided Student's t-tests were applied. For multiple comparisons, one-way ANOVA was conducted, followed by Tukey's post hoc test (to compare all possible combinations) or Dunnett's post hoc test (to compare data to one selected group).

All numerical data are presented as the average ± SEM unless otherwise stated. The *n* number in the figure legends denotes the number of biological replicates. The levels of significance (p value) are indicated in figure captions.

Results

Silencing of PARP-2 inhibits autophagy

Silencing of PARP-2 inhibits autophagy in immortalized myoblast cells

Control and PARP-2 silenced C2C12 myoblast cells were studied by electron microscope. Analyzing the cells by electron microscope, we found cytosolic electron-dense particles in PARP-2 silenced cells that looked like late-stage autophagic vesicles. To provide evidence that the electron-dense structures were indeed of autophagic nature, we studied the expression of LC3 protein which is a well-known and widely used autophagy marker. We examined the LC3 level in scPARP2 and shPARP2 C2C12 cells by Western blotting and immunofluorescence experiments. We detected increased LC3 expression in PARP-2 silenced cells compared to the control cells, and increase in the number of LC3-positive vesicles was observed on immunofluorescence images. The increase in the level of LC3 expression mainly affected the lipidated, autophagic membrane-associated form of LC3, LC3-II. The appearance of LC3 dots suggests increased activity of autophagy in the absence of PARP-2. To support our previous results, LC3 expression was also examined in C2C12 cells in which PARP-2 was transiently silenced. Acute silencing of PARP-2 by siRNA, similar to the established cell line, the LC3 expression increased.

Studying the process of autophagy, the next step was to label the cells with LysoTracker Deep Red fluorescent dye, which accumulates in acidic vesicles (lysosome, autolysosome), thus labeling them. Assessment of the confocal microscopy images, the number of LysoTracker-positive organelles increased in PARP-2 silenced cells, which also indicates increased activity of autophagic.

Next, we studied how scPARP2 and shPARP2 C2C12 cells respond to known modulators of autophagy, and whether different changes in LC3 expression were observed in the control and PARP-2 silenced cell lines, respectively. Cells were treated with chloroquine and fasted, and then we examined the change in the number of LC3-positive vesicles by immunofluorescence. We found that both interventions led to increased LC3 expression in both cell lines. These data suggest that, while both cell lines respond to starvation by increased formation of autophagic vesicles, basal autophagic flux

is partially impaired in PARP-2 silenced cells based on the similar numbers of LC3-positive vesicle seen in the two cell lines upon treatment with chloroquine.

Induction of autophagy depends on the induction of SIRT1 and the inhibition of AMPK

We assessed how the major energy sensors, mTOR kinase complexes and AMPK, change in the absence of PARP-2. Based on our Western blotting experiments, AMPK expression did not change, but its auto-phosphorylation status decreased in the absence of PARP-2. We examined mTORC1 and mTORC2 activity through the expression and phosphorylation state of the proteins they can regulate (p70 S6 kinase and Akt). MTORC1 activity, measured through assessing the phosphorylation of the p70 S6 kinase, did not change in shPARP2 C2C12 cells, whereas mTORC2 activity, assessed through the phosphorylation of Akt kinase, showed mild reduction. These results confirm our observation that the silencing of PARP-2 led to profound changes in cellular energy sensing.

We assessed whether these changes are functional by using pharmacological modulators of the above energy sensors, we studied whether these modulators could influence the increased LC3 expression that appears upon silencing of PARP-2. AICAR, an activator of AMPK, reduced the proportions of the high LC3 expression in PARP-2 silenced cells compared to the control cells. Rapamycin, an inhibitor of mTORC1, increased the LC3 expression in control cells. Olaparib as a PARP inhibitor and nicotinamide riboside (NR) as a NAD^+ precursor decreased the number of LC3-positive vesicles, however, none of these modulators could reduce the increased LC3 level upon silencing of PARP-2, to baseline (control, untreated cells).

In our next study, we sought to answer whether the increased SIRT1 expression and activity observed after silencing of PARP-2 was related to the increased LC3 expression in the absence of PARP-2. SIRT1 activation can induce autophagy, its role of autophagy is previously described in the literature. Resveratrol was used for pharmacological activation of SIRT1, while we used EX-527 to inhibit SIRT1, and we examined the LC3 expression in scPARP2 and shPARP2 C2C12 cells. We found that resveratrol increased LC3 expression in both cell lines, while inhibition of SIRT1 by EX-

527 reduced the number of LC3-positive vesicles that increased upon silencing of PARP-2.

To confirm the changes during pharmacological inhibition, SIRT1 was transiently silenced by siRNA in scPARP2 and shPARP2 C2C12 cells. After silencing of SIRT1, we observed a decrease in the number of LC3-positive vesicles, similar to we found if SIRT1 was inhibited by pharmacologically with EX-527. These results suggest that increased SIRT1 activity upon silencing of PARP-2 has a positive effect on autophagy.

The number of LC3-positive vesicles increase in primary cells upon the genetic deletion of PARP-2

To confirm our previous experimental results, we also studied the expression of LC3 protein in mouse primary MEF cells by immunofluorescence. A primary cell line from a PARP-$2^{+/+}$ mouse was compared with 5 different PARP-2 knockout mice (PARP-$2^{-/-}$) origin primary cell lines. Assessment of confocal microscopy images, we showed increase in the number of LC3-positive vesicles in PARP-$2^{-/-}$ MEF cells compared to the PARP-$2^{+/+}$ MEF cell, which is in good agreement with our previous observation that we found in immortalized C2C12 cells. This result suggests that autophagic activity enhanced in the absence of PARP-2, but also suggests that the increased LC3 expression is not due to immortalization.

The activity of PARP-2 plays a role in mediating autophagy

We studied the activity of mTOR complexes and AMPK in scPARP2 and shPARP2 C2C12 cells that we treated with PARP inhibitors, olaparib or PJ34. When we treated cells with these inhibitors, we found that the phosphorylation of AMPK decreased in both the control and PARP-2 silenced cell lines. We observed increased mTORC1 activity, measured through assessing the phosphorylation of the p70 S6 kinase, in scPARP2 C2C12 cells, whereas when we used PARP inhibitors, we detected in a decrease in mTORC1 activity in PARP-2 silenced cells. Inhibition of PARP activity reduced the phosphorylation of Akt kinase in control cells, however, it had no effect in

PARP-2 silenced cells. These results confirmed our hypothesis that decreased PARP-2 activity affects cellular energy sensor system.

Silencing of PARP-2 causes fragmentation of the mitochondrial network and increases mitochondrial ROS production

Silencing of PARP-2 leads to fragmented mitochondria

We studied the mitochondrial structure in control and PARP-2 silenced C2C12 myoblast cells by immunofluorescence. We labeled mitochondria with MitoTracker Red fluorescent dye, which accumulates in the mitochondria of living cells. Moreover, to study the mitochondrial morphology by confocal microscope, Tomm20 was labeled with a specific antibody. We found a fragmented mitochondrial structure in PARP-2 silenced cells at both labeling. We used ImageJ software's Mito-Morphology macro and we found that the mitochondrial content and circularity increased, while mitochondrial perimeter and form factor decreased in the absence of PARP-2, which also suggests that the mitochondrial network fragmented.

As the next step, we examined the scPARP2 and shPARP2 C2C12 cells by electron microscope. We found that the number of mitochondrial cross-sections increased in the absence of PARP-2, which also indicates the fragmentation of mitochondria.

To confirm our previous results, we also examined the mitochondrial structure in PARP-2 transiently silenced C2C12 cells. Acute silencing of PARP2 also led to the fragmentation of mitochondria, similar to the observations in the established cell line. We detected increased mitochondrial content and circularity and decreased mitochondrial perimeter and form factor.

SIRT1 activation, mitophagy and the deregulation of the mitochondrial fusion–fission system are not involved in mitochondrial fragmentation in PARP-2 silenced cells

Multiple parallel pathways can influence the mitochondrial network integrity. The selective degradation of mitochondria, called mitophagy, can also cause the fragmentation of the mitochondrial network, so we studied the possibility of this process. To confirm this, in scPARP2 and shPARP2 C2C12 cells, we used markers that were well described in previous experiments to examine the localization of mitochondria and

autophagosomes: mitochondria were labeled with MitoTracker Red fluorescent dye or Tomm20 antibody, while autophagosomes were labeled with LC3 antibody. Confocal microscopy images were evaluated using the EzColocalization plugin of ImageJ software, during which we could not detect any overlap between autophagosomes and mitochondria.

In our next experiment, we examined the effect of the increased SIRT1 activity on mitochondrial network in the absence of PARP-2. SIRT1 was acute silenced in scPARP2 and shPARP2 C2C12 cells, and the mitochondrial structure was examined with MitoTracker Red fluorescent dye. We did not detect any changes during the assessment of the confocal microscopy images, suggesting that the mitochondrial fragmentation in the absence of PARP-2 is not the result of the increased SIRT1 activity. Silencing of SIRT1 had no effect on the mitochondrial morphology, and the fragmented mitochondrial structure upon silencing of PARP-2 could not be compensated by silencing of SIRT1, so mitochondria did not reverse the changes to mitochondrial morphology.

The next step was to study the mRNA and protein expression of proteins involved in mitochondrial fusion or fission and mitophagy (Mfn1, Mfn2, Opa1, Fis1, Drp1, Parkin, and Pink1) in control and PARP-2 silenced C2C12 cells. We did not detect any changes in the expression of these proteins upon silencing of PARP-2 to be consistent with that observed in shPARP2 HepG2 cells.

Mitochondrial fragmentation upon silencing of PARP-2 is not specific for C2C12 myoblasts

After to study the mitochondrial network in myoblasts, we assessed another cell line in which PARP-2 was silenced. So we examined, our observations are specific for the myoblast cells or similar in other cell types. In scPARP2 and shPARP2 HepG2 cells, similar to C2C12 cells, mitochondria were labeled with MitoTracker Red fluorescent dye and Tomm20 antibody, respectively. Similar to myoblast cells, a fragmented mitochondrial network was detected in PARP-2 silenced HepG2 cells at both labels.

In our next experiment, scPARP2 and shPARP2 HepG2 cells were examined by electron microscope. Electron micrographs showed increase in the number of

mitochondrial cross-sections in the absence of PARP-2, which also indicates fragmentation of the mitochondrial structure.

In the next experiment, we sought to answer whether the altered mitochondrial structure in PARP-2 silenced HepG2 cells is associated with mitophagy or change in mitochondrial dynamics. We first examined autophagosome–mitochondrial co-localization in scPARP2 and shPARP2 HepG2 cells by confocal microscope with two types of mitochondrial labeling, and then the expression of proteins involved in mitochondrial fusion–fission was studied. Similarly to C2C12 cells, we could not detect co-localization signal between autophagosomes and mitochondria, and proteins involved in mitochondrial fusion–fission and mitophagy (Mfn1, Mfn2, Opa1, Fis1, Drp1, Parkin and Pink1) did not change that could have explained the mitochondrial fragmentation upon silencing of PARP-2.

Silencing of PARP-2 leads to oxidative stress

The redox state of cells changes in response to PARP inhibition, described in several cases, so we decided to study oxidative and nitrosative stress markers in scPARP2 and shPARP2 C2C12 cells. Superoxide production was first examined by dihydroethidium, which increased, and then we detected increase in lipid peroxidation by determining thiobarbituric acid-reactive products. The expression of 4HNE-modified proteins was increased in the absence of PARP-2, which indicates increased oxidative effect in cells. As a marker of nitrosative stress, we detected a slightly increased iNOS expression, and then we studied the expression of NTyr by Western blotting, in which a slight increase was observed in the absence of PARP-2. We found decreased expression of Nox4, suggesting that the source of the increased ROS production is not the NADPH oxidase. Our results show that the redox homeostasis of cells is altered in the absence of PARP-2 and oxidative stress induced.

Next, we wanted to clarify whether mitochondrial fragmentation in the absence of PARP-2 and increased ROS production are related. Control and PARP-2 silenced myoblast cells were treated with general reductants, GSH, NAC and MitoTEMPO, respectively, and then we examined the changes in mitochondrial morphology with MitoTracker Red fluorescent dye. We could not detect any changes in mitochondrial

content upon antioxidant treatment; however, the altered mitochondrial morphology in the absence of PARP-2 could be reduced by treatment with these antioxidants. We detected increase in mitochondrial perimeter and form factor, while circularity decreased, suggesting that mitochondrial fragmentation induced by PARP-2 deficiency is based in part on oxidative stress with mitochondrial origin.

In our next experiment, we assessed how the expression of mitochondrial genes responds to the previously used antioxidant treatment. The expression of these genes has previously been shown to increase in the absence of PARP-2. Therefore, control and PARP-2 silenced C2C12 cells were treated with antioxidants and then the expression of Ndufa2, Ndufb3, Ndufb5, COX17, and cyt C was examined using RT-qPCR. Significant difference in the expression of the tested proteins was detected in the untreated control and PARP-2 silenced cells, which differences were reduced upon the antioxidant treatment.

The effect of antioxidants on the oxygen consumption rate and the fatty acid oxidation was examined with a Seahorse analyzer, while the ATP content was determined by fluorescence measurement. We found decrease in both basal oxygen consumption rate and fatty acid oxidation and ATP content in control and PARP-2 silenced C2C12 cells. The increased oxygen consumption rate, fatty acid oxidation, and ATP levels, which appear upon silencing of PARP-2, were reduced upon antioxidant treatment.

Based on our results, GSH and NAC reduced the proliferative capacity of cells, while MitoTEMPO had no effect on this process, so we found no difference between scPARP2 and shPARP2 cells. In addition, neither antioxidants nor silencing of PARP-2 did not effect on the spontaneous cell death.

PARP-1 and PARP-3 are not involved in mitochondrial fragmentation elicited by the silencing of PARP-2

Previous studies have demonstrated that the activity of PARP-1 and PARP-3 were implicated in regulating mitochondrial morphology. In our next experiments, we examined whether PARP-1 and PARP-3 were associated with a change in mitochondrial morphology in the absence of PARP-2.

Firstly, we started to study the effect of PARP-1 on mitochondrial structure by transient silencing of PARP-1 in control and PARP-2 silenced C2C12 myoblast cells. The Western blotting image of PAR shows that the acute silencing of PARP-1 reduced the autoPARilation of PARP-1. Mitochondrial morphology was studied with MitoTracker Red fluorescent dye in the same cells. During the evaluation of the fluorescence images, a slight fragmentation of the mitochondrial structure was detected in the control cells in PARP-1 deficiency; however, the altered mitochondrial morphology in PARP-2 deficiency was not affected by the silencing of PARP-1.

PARP-3 was transiently silenced in scPARP2 and shPARP2 C2C12 cells, then we examined the PARylation process. Two siRNAs targeting PARP-3 showed an increase in the PAR Western blotting image, suggesting increased PARP-1 activity. Mitochondrial morphology was examined with MitoTracker Red fluorescent dye in the same cells, and then, in the analysis of the fluorescent images, similar to PARP-1, there was no change in either control or PARP-2 silenced cells.

Discussion

The basal autophagic flux is partially impaired upon silencing of PARP-2

Autophagy is an intracellular degradation process characteristic of all eukaryotic cells, its main function is cytoprotection, in which cell eliminates its own substances, degrades its own organelles or macromolecules. Moreover, autophagy also plays an essential role in the protection against intracellular pathogens. The main function of autophagy is the cytoprotection, in which cell degrades its own organelles or macromolecules. The main regulators of autophagy are the members of energy sensor system, AMPK and mTOR kinase complexes. Autophagy is present in all cell types, disorders of autophagy can lead to pathophysiological conditions. The role of PARP-1 and PARP-10 is already known in the process of autophagy, however, the role of PARP-2 has not yet been identified in autophagy.

Autophagy was studied in myoblast cells in which PARP-2 was silenced using shRNA. These cells generated for a previous study. Based on our experimental results, silencing of PARP-2 increased the number of autophagic vesicles, which manifested by increased LC3 expression in the absence of PARP-2. This result was also confirmed during the acute silencing of PARP-2 by siRNA. In our next experiment, we sought to answer how the control and PARP-2 silenced cell lines respond to treatment with substances capable of modifying the autophagy process, and whether different changes may be observed in the two cell lines. Chloroquine as an autophagy inhibitor inhibits the process of lysosomal degradation by neutralizing lysosomal pH, whereas serum starvation initiates autophagy through regulation of the mTOR complex. Increased LC3 expression was also detected in control and PARP-2 silenced cell lines after chloroquine treatment and serum starvation, however, the difference in LC3 signal between the two cell lines observed in the absence of PARP-2 was reduced by both treatments, suggesting that, the absence of PARP-2 primarily inhibits the basic autophagic activity. Based on the literature, serum depletion reduces PARP-2 expression, mRNA expression of PARP-2 is not altered during serum starvation, but proteosomal degradation of PARP-2 is faster.

According to our research, depletion of PARP-2 modifies the energy sensor system of cells: we found decreased AMPK and mTORC2 activity in the absence of

PARP-2. The role of AMPK is known in the process of autophagy, and based on the literature, AMPK is an inducer of autophagy. However, our results suggest that activation of AMPK by AICAR partially blocks the increased autophagic activity that appeared upon silencing of PARP-2. This suggests that a decrease in AMPK activity after PARP-2 silencing contributes to an increase in autophagic activity. While mTORC1 activity is not affected by PARP-2 deficiency, a slight decrease in mTORC2 activity was detected in shPARP2 cells. Both mTORC1-independent and mTORC2-dependent forms of autophagy have been previously identified, in which case phosphorylation of Akt by mTORC2 does not alter the activity of mTORC1. The relationship between PARP-2 and the members of mTOR complex has not yet been described. However, we have a research that investigates the interaction between PARP-2 and mTOR complexes. Based on our preliminary results, the decreased activity of mTORC2 can be attributed to the decreased expression of the Rictor protein in the mTORC2 complex, which has also been previously shown to be PARylatable.

The regulation of autophagy is a complex process in which, in addition to the main regulators, SIRT1 also plays a role. It has previously been observed that SIRT1 activation induces induces autophagy through LC3-I–LC3-II conversion and enhanced autophagosome formation. SIRT1 can affect autophagy both directly and indirectly way. On the one hand, it is able to directly interact and deacetylate the key proteins involved in the mechanism of autophagy, Atg5, Atg7, and Atg8. On the other hand, SIRT1 indirectly regulates the autophagy by deacetylation of FOXO transcription factors. Deacetylated FOXO1 by SIRT1 stimulates the formation of autophagosomes by inducing transcription of Rab7, whereas deacetylated FOXO3 induces autophagy by stimulating transcription of Bnip3 and NIX receptors. Induced autophagy by activation of SIRT1 has cardioprotective and neuroprotective effects. Based on our results, induction of autophagy during PARP-2 depletion is also associated with increased activation of SIRT1 in the absence of PARP-2. Silencing of PARP-2 increases SIRT1 activity because PARP-2 is a repressor of the SIRT1 promoter, and thus increased expression and activity of SIRT1 is observed in the absence of PARP-2. Inhibition of autophagy by 3-MA decreases the expression of SIRT1, however, resveratrol can induce autophagy in a SIRT1-dependent manner. However, it is important to highlight that resveratrol may induce autophagy in a SIRT1 and mTOR-

independent manner through direct inhibition of S6 kinase. Moreover, the intracellular concentration of NAD^+ also has an effect on the autophagy process by influencing the activity of SIRT1 and PARP-1 enzymes, as the activity of both enzymes is regulated by NAD^+ concentration. In our experiments, when we treated cells with NR, a NAD^+ precursor, we found that the NAD^+ level increased and it was able to reverse the LC3-positive vesicle accumulation upon silencing of PARP-2. Pharmacological inhibition and gene silencing of SIRT1 also reduced the number of autophagic vesicles and it consistent with the literature data.

Based on our results, it can be concluded that silencing of PARP-2 increases the number of LC3-positive autophagic vesicles by modulating cellular energy sensor system, AMPK and mTORC2, and SIRT1, respectively.

Silencing of PARP-2 causes mitochondrial fragmentation and induces oxidative stress

As the second part of our research, we studied the relationship between PARP-2 and mitochondria. The relationship between PARP-2 and mitochondria has been previously identified in several cases, however, in our research, we wanted to expand our knowledge of their relationship. Increased SIRT1 activation also plays a role in the process of mitochondrial biogenesis in the absence of PARP-2. Oxidative phenotype can be detected in the skeletal muscle and liver of PARP-2 knockout mice, in addition to increased mitochondrial content and mtDNA levels.

The mitochondrial structure was studied in mouse C2C12 cells in which PARP-2 was silenced by shRNA technique, and we confirmed our results in human HepG2 cells and during the acute silencing of PARP-2. Based on our results, silencing of PARP-2 modified the mitochondrial network and can cause fragmentation of the mitochondrial structure. In addition, we found an increase in the mitochondrial content upon silencing of PARP-2, which correlates with previous research and suggests mitochondrial biogenesis. The other major PARP enzyme, PARP-1, has previously been shown to affect the mitochondrial network integrity.

Multiple parallel pathways can cause fragmentation of the mitochondrial network, including selective degradation of mitochondria by autophagy, called

mitophagy, dysfunction of the mitochondrial dynamic, alteration or complete absence of expression of proteins involved in the process, or SIRT1 activation. Based on the literature, inhibition of PARP-1 may cause mitophagy, as hyperactivation of PARP-1 results decreased NAD^+ levels and consequently SIRT1 activity, which enhances Pink1 activation and causes mitochondrial hyperpolarization. SIRT1 affects the mitochondrial network because increased SIRT1 activity causes an increase in mitochondrial content and fragmentation of the mitochondrial network. Based on our research, moreover, we did not detect any changes in the process of mitochondrial dynamics that would have explained the disintegration of the mitochondrial network and similar changes in both C2C12 and HepG2 cell lines, nor did we show increase in activity of mitophagy. Our results suggest that none of the studied processes is responsible for the altered mitochondrial network in the absence of PARP-2.

In our next experiment, oxidative stress was appeared upon silencing of PARP-2. Based on the literature, genetic or pharmacological inhibition of PARP may alter the redox state of cells. In our experiments, PARP-2 depletion showed an increase in lipid peroxidation in oxidative stress markers, including superoxide production detected by hydroethidine staining, and expression of 4HNE-modified proteins, and in the formation of thiobarbituric acid-reactive products in TBARS. They suggest increased oxidative stress in the absence of PARP-2. After treatment with antioxidants, GSH and NAC, and the mitochondrial-targeted antioxidant, MitoTEMPO, we found that antioxidants did not affect mitochondrial biogenesis in the absence of PARP-2, or did not alter mitochondrial content, but they affect the mitochondrial network, however, they affect the altered mitochondrial network in the absence of PARP-2. The fragmented mitochondrial structure that was detected in the absence of PARP-2 restored after treatment with antioxidants, and a phenotype characteristic of control cells appears. It has been shown in many cases that increased ROS production causes fragmentation of mitochondria. Changes in mitochondrial dynamics are closely related to redox homeostasis in cells. MitoTEMPO has previously been shown to affect mitochondrial morphology. Silencing of PARP-2 increased the expression of some members of the mitochondrial respiratory chain such as Ndufa2, Ndufb3, Ndufb5, COX17, and cyt C, which was reduced by antioxidants. According to our experimental results, the antioxidants were able to reduce

their increased expression in the absence of PARP-2. In addition, after treatment with antioxidants, the oxygen consumption, the fatty acid oxidation, and consequently the ATP content of the cells in the control and PARP-2 silenced cells decreased, but the differences between the untreated control and PARP-2 cells did not change. Among the antioxidants, GSH and NAC, correlated with the literature, reduced the ability of the cells to proliferate, however, control and PARP-2 silenced cells responded equally after antioxidant treatment, with no difference between the two cell lines. In addition, neither antioxidant treatment nor PARP-2 silencing showed an effect on spontaneous cell death. These results suggest that the source of the increased ROS production in the absence of PARP-2 is in part due to fragmentation of mitochondria and mitochondrial structure caused by oxidative stress induced by increased ROS production.

Two members of the PARP family, PARP-1 and PARP-3, have previously been shown to be able to influence the mitochondrial morphology. Pharmacological inhibition of PARP-1 reduces the mRNA level of Opa1 protein involved in the mitochondrial fusion process, resulting in a more fragmented mitochondrial network after treatment with olaparib. Inhibition of PARP-3 has also been associated with increased ROS production, the main origin of which is the mitochondrial NADPH oxidase. However, according to our studies, silencing of PARP-1 or PARP-3 did not cause morphological changes in the mitochondrial structure, so the mitochondrial fragmentation in PARP-2 silenced cells was not affected by silencing of PARP-1 or PARP-3.

Based on these results, we can conclude that the absence of PARP-2 causes fragmentation of the mitochondrial network and is associated with increased ROS production, which induces oxidative stress in cells. In the absence of PARP-2, the oxidative state of cells changes, leading to mitochondrial biogenesis and the development of oxidative stress causing fragmentation of the mitochondrial structure.

Summary

PARP-2, a member of the PARP family, performs the post-translational modification of proteins. PARP-2 enzyme is involved in the regulation of several metabolic processes. In our work, we investigated the relationship between PARP-2 and autophagy and the role of PARP-2 in mitochondrial homeostasis.

In the absence of PARP-2, basal autophagy is disrupted and the number of LC3-positive vesicles increases in cells. The silencing of PARP-2 rearranged the energy homeostasis of cells, disrupting autophagy. The disruption of autophagy is due to decreased activity of AMPK and mTORC2 and increased activity of SIRT1. The altered energy homeostasis affects autophagy. Pharmacological inhibition of AMPK by AICAR and pharmacological or genetic silencing of SIRT1, and the cause of NR as NAD^+ precursor, reduced the expression of LC3, these compounds are able to reduce the increased accumulation of LC3-positive vesicle in the absence of PARP-2.

Subsequently, we showed that silencing of PARP-2 causes fragmentation of the mitochondrial network and the induction of mitochondrial biogenesis. There are several pathways that can cause changes in integrity of the mitochondrial network, among others, mitophagy, altered expression or dysregulation of the mitochondrial fusion–fission machinery, and activation of SIRT1. However, in our experiments, we did not find any major change in these processes that would justify changes in the mitochondrial structure in the absence of PARP-2. Silencing of PARP-1 or PARP-3 did not cause morphological changes in mitochondrial structure, the fragmentation observed in PARP-2 silenced cells was not affected by silencing of either enzyme. Upon the silencing of PARP-2, the expression of oxidative stress markers as superoxide production, expression of 4HNE-modified proteins or formation of thiobarbituric acid-reactive products increased. Treatment of cells with thiol reductants, GSH and NAC, and the mitochondrial-targeted antioxidant, MitoTEMPO, did not change the mitochondrial content, but reverted mitochondrial fragmentation. We conclude that the altered mitochondrial phenotype that appears in the absence of PARP-2 is associated with increased level of the oxidative stress causing the fragmentation of the mitochondrial structure.

List of publication

UNIVERSITY of DEBRECEN

UNIVERSITY AND NATIONAL LIBRARY
UNIVERSITY OF DEBRECEN
H-4002 Egyetem tér 1, Debrecen
Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

Registry number: DEENK/459/2021.PL
Subject: PhD Publication List

Candidate: Laura Jankó
Doctoral School: Doctoral School of Molecular Medicine
MTMT ID: 10069221

List of publications related to the dissertation

1. **Jankó, L.**, Kovács, T., Laczik, M., Sári, Z., Ujlaki, G., Kis, G., Horváth, I., Antal, M., Vígh, L., Bálint, B. L., Uray, K., Bai, P.: Silencing of Poly(ADP-Ribose) Polymerase-2 Induces Mitochondrial Reactive Species Production and Mitochondrial Fragmentation.
   Cells. 10 (6), 1-23, 2021.
   DOI: http://dx.doi.org/10.3390/cells10061387
   IF: 6.6 (2020)
2. **Jankó, L.**, Sári, Z., Kovács, T., Kis, G., Szántó, M., Antal, M., Juhász, A. G., Bai, P.: Silencing of PARP2 blocks autophagic degradation.
   Cells. 9 (2), 1-21, 2020.
   DOI: http://dx.doi.org/10.3390/cells9020380
   IF: 6.6

List of other publications

3. Sári, Z., Mikó, E., Kovács, T., **Jankó, L.**, Csonka, T., Lente, G., Sebő, É., Tóth, J., Tóth, D., Árkosy, P., Boratkó, A., Ujlaki, G., Török, M., Kovács, I., Szabó, J., Kiss, B., Méhes, G., Goedert, J. J., Bai, P.: Indolepropionic Acid, a Metabolite of the Microbiome, Has Cytostatic Properties in Breast Cancer by Activating AHR and PXR Receptors and Inducing Oxidative Stress.
   Cancers (Basel). 12 (9), 1-27, 2020.
   DOI: http://dx.doi.org/10.3390/cancers12092411
   IF: 6.639
4. Sári, Z., Mikó, E., Kovács, T., Boratkó, A., Ujlaki, G., **Jankó, L.**, Kiss, B. K., Uray, K., Bai, P.: Indoxylsulfate, a Metabolite of the Microbiome, Has Cytostatic Effects in Breast Cancer via Activation of AHR and PXR Receptors and Induction of Oxidative Stress.
   Cancers (Basel). 12 (10), 1-23, 2020.
   DOI: http://dx.doi.org/10.3390/cancers12102915
   IF: 6.639

UNIVERSITY of DEBRECEN

UNIVERSITY AND NATIONAL LIBRARY
UNIVERSITY OF DEBRECEN
H-4002 Egyetem tér 1, Debrecen
Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

5. Hegedűs, C., Boros, G., Fidrus, E., Kis, G., Antal, M., Juhász, T., Janka, E. A., **Jankó, L.**, Paragh, G. J., Emri, G., Bai, P., Remenyik, É.: PARP1 Inhibition Augments UVB-Mediated Mitochondrial Changes-Implications for UV-Induced DNA Repair and Photocarcinogenesis. *Cancers (Basel). 12* (1), 1-29, 2020.
DOI: http://dx.doi.org/10.3390/cancers12010005
IF: 6.639

6. Kovács, P., Csonka, T., Kovács, T., Sári, Z., Ujlaki, G., Sipos, A., Karányi, Z., Szeőcs, D., Hegedűs, C., Uray, K., **Jankó, L.**, Kiss, M., Kiss, B. K., Laoui, D., Virág, L., Méhes, G., Bai, P., Mikó, E.: Lithocholic acid, a metabolite of the microbiome, increases oxidative stress in breast cancer.
Cancers (Basel). 11, 1-31, 2019.
IF: 6.126

7. Márton, J., Péter, M., Balogh, G., Bódi, B., Vida, A., Szántó, M., Bojcsuk, D., **Jankó, L.**, Bhattoa, H. P., Gombos, I., Uray, K., Horváth, I., Török, Z., Bálint, B. L., Papp, Z., Vígh, L., Bai, P.: Poly(ADP-ribose) polymerase-2 is a lipid-modulated modulator of muscular lipid homeostasis. *Biochim. Biophys. Acta. Mol. Cell Biol. Lipids. 1863* (11), 1399-1412, 2018.
DOI: http://dx.doi.org/10.1016/j.bbalip.2018.07.013
IF: 4.402

Total IF of journals (all publications): 43,645
Total IF of journals (publications related to the dissertation): 13,2

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

08 October, 2021

DEBRECENI EGYETEM * Egyetemi és Nemzeti Könyvtár *