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

Exploring the diverse cellular functions of the proteasome activator PA200

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DEBRECEN, 2021

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The Examination takes place at the Department of Biophysics and Cell Biology, Faculty of Medicine. University of Debrecen, 21st of June, 2018, 11:00 AM

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INTRODUCTION

The proteasome activator, PA200 is one of the activator subunits of the proteasomes. PA200 binds to the 20S proteasome core and enhances degradation of peptides in a ubiquitin and ATP independent manner. PA200 was identified at the first time in bovine testis, then its homologs were described in worm (*Caenorhabditis elegans*), yeast (*Saccharomyces cerevisiae*), and plants (*Arabidopsis thaliana*). Bioinformatics analysis of PA200 amino acid sequence revealed that PA200 entirely consists of 32 HEAT-Like motif repeats, which form at the tertiary structure an asymmetric dome-like structure with major and minor lobes. At the protein and mRNA levels, three PA200 isoforms were identified in eukaryotic cells; PA200-I; ~ 190 kDa, PA200-II; ~ 140 kDa and, PA200-III; ~ 130 kDa. Only PA200 I isoform can bind to the 20S proteasome core to form single (PA200-20S) or double capped (PA200-20S-PA200) proteasome complexes. Biochemical characteristics of PA200 are fully understood, however, to date, the biological functions of PA200 are more controversial. PA200 has structural and functional particularities that candidate this protein to have other important functions in addition to its role in proteolysis machinery. PA200 is entirely reached with HEAT-like motif repeats which are typically associated with protein-protein recognition, suggesting that PA200 may have a role as an adaptor protein. Furthermore, PA200 binds to 20S and enhances the axial gate opening like all other proteasome activators, however, the mechanism of substrate delivery into 20S catalytic chamber remains unclear. Additionally, it has been reported that the affinity of PA200 to 20S proteasomes is the lowest with a percentage of 1-2% compared to 20S-19S (15-25%) and 20S-11S (5-10%) proteasome complexes in different cell types. Notably, some cell types do not show the interaction of PA200 and the 20S proteasome, such as cardiac muscle cells in mice. This suggests that PA200 may have other functions than an activator of the ubiquitin-proteasome system (UPS) that needs further investigation.

The first proposed biological function of PA200, in addition to its role as proteasome activator, was that the protein is involved in repair of DNA double-strand breaks (DSBs). However, the exact molecular mechanisms of PA200 in the DNA DSBs repair process remain unclear. PA200 knockout mice show a defect in male fertility but not females, and this phenomenon was explained as an impairment in the DNA DSBs repair machinery in meiotic cells upon deletion of PA200 during spermatogenesis. The authors argue that if we consider PA200 as an essential factor in

DNA DSBs repair, we expect to see the same phenotypes of mice-deficient of well-known DNA DSBs repair factors in PA200$^{\Delta/\Delta}$ mice. Deletion of these factors leads to both male and female sterility, which was not observed in the PA200$^{\Delta/\Delta}$ female mice. In the same context, it has been reported that proteasome activity in sperm of PA200$^{\Delta/\Delta}$ mice is strongly reduced only in the double knockout (dKO) mice of *PSME3* and *PSME4* (encoding for PA28γ and PA200, respectively), but not a single *PSME4* KO mouse. It is known that PA200 is highly expressed in testis compared to all other tissues and its deletion did not show any major effect on proteasome activity, unlike PA28γ. This suggests that PA200 influences the spermatogenesis process with another mechanism that is not related to the proteolytic machinery. It has been shown that loss of Blm10 (yeast ortholog of PA200) leads to impaired mitochondrial respiration and an increase in oxidative stress sensitivity. More interestingly, Blm10 expression level increased upon switching from fermentation to oxidative metabolism, which suggests the possible role of PA200 in oxidative phosphorylation machinery and overall metabolism. Furthermore, recent studies demonstrated that PA200 knockout mice exhibit muscle fiber atrophy and accelerated aging. The accelerated aging in PA200-deficient mice could be related to mitochondrial dysfunction, which is a common factor of aging and aging-related neurodegenerative disorders.

Almost all the above-discussed functions of PA200 were related to proteasome activity without providing an exact molecular mechanism behind it. Furthermore, the possible role of PA200 in the pathophysiology of diseases through the maintenance of cell survival, mitochondrial integrity, and metabolism is undetermined. In the present study, we investigated the possible new cellular functions of PA200, in the SH-SY5Y cell line, using chromatin immunoprecipitation (ChIP) followed by ChIP-sequencing (ChIP-seq) analysis and global transcriptomic analysis approaches. My main research work focused on the effects of PA200 on cell survival, mitochondrial integrity, and metabolism under normal condition and upon mitochondrial stress.

AIMS

Our main goal in the present study is to reveal new cellular functions of PA200, an alternative activator of the proteasome.

First, we planned to determine the effects of PA200 on cell viability, necrosis, and apoptosis under normal conditions and stressed mitochondria using different mitochondrial inhibitors (rotenone, oligomycin, antimycin A). Furthermore, since PA200 is a chromatin component and translocated to the nucleus upon irradiation exposure, we aimed to investigate the possible role of PA200 in the transcriptional machinery under normal conditions and upon mitochondrial stress using chromatin immunoprecipitation (ChIP) and ChIP-seq approaches supported by RT-qPCR analysis. Second, we aimed to reveal new promising functions of PA200 by investigating the global transcriptomic pattern upon depletion of PA200 using RNA-seq approach and functional gene category analysis supported by functional validations. Deletion of the PA200 ortholog in yeast (Blm10) leads to mitochondrial dysfunction, thus we speculated that this function is conserved in mammals; we aimed to investigate mitochondrial activity, biogenesis, and dynamics.

The obtained data may provide more information on the interplay between the proteasome activator, PA200, cell survival, and mitochondrial integrity, and might contribute to develop therapies for aging-related diseases, such as neurodegenerative diseases.

MATERIALS AND METHODS

Cell culture

Human neuroblastoma (SH-SY5Y) and Human embryonic kidney 293 (HEK293T) cell lines (European Tissue Culture) were maintained in DMEM-high glucose supplemented with 10 % heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin in 5% CO_2 humidified incubator at 37 °C.
Lentiviral technology was used to downregulate the expression of *PSME4*/PA200 in the SH-SY5Y neuroblastoma cell line.

Downregulation of *PSME4*/PA200

Lentiviral technology was used to generate stable *PSME4*/PA200 depleted cells in the SH-SY5Y neuroblastoma cell line. First, the virus soup was produced in HEK 293T cells. HEK 293T cells were co-transfected with the packaging-enveloping vectors and the expression plasmids pGIPZ-GFP containing the shRNA target sequences. pGIPZ-GFP plasmids containing non-silencing shRNA sequences were used as a control. The packaging-enveloping vectors and the expression plasmids were co-transfected at a 1:1 ratio using Lipofectamine 3000 according to the manufacturer's protocol. The transduction of human SH-SY5Y neuroblastoma cells was assessed as the following protocol, SH-SY5Y cells were seeded in 24 well plates at 60% confluency in DMEM high-glucose. On the day of transduction (80% confluency), cells were incubated with virus-containing antibiotic-free media supplemented with 8 µg/ml polybrene, in a 5% CO_2 humidified incubator at 37°C. After 72h of viral transduction, the culture medium was supplemented with 1.25 µg/ml puromycin for selection. The puromycin selected and amplified cells were further analyzed by RT-qPCR and western blot to verify the depletion of *PSME4*/PA200.

Chromatin Immunoprecipitation

ChIP assay was assessed in SH-SY5Y cells under physiological conditions and upon treatment with mitochondrial inhibitors for 3h. Formaldehyde, at a final concentration of 1%, in culture medium was used to cross-link the cells for 10 min at room temperature (RT). 125 mM glycine was applied for 10 min at RT to quench the cross-linking. Cells were suspended in lysis buffer and sonicated twice, 5 cycles for 30 s on/off with maximum level. Antibodies against PA200 proteasome activator and H3K27ac were used for immunoprecipitation. The protein-DNA complex was elution by adding 400 µl of elution buffer (1 % SDS and 0.1 M NaHCO3) and decross linked by adding NaCl at a final concentration of 0.2 M, followed by shaking overnight at 65 °C. DNA was purified using the PCR Clean-up Kit according to the manufacturer's protocol. ChIP-seq analysis was performed in Galaxy version 18.01.rc1 using data released by NextSeq 500 System from Illumina, Center for Clinical Genomics and Personalized Medicine, Core Facility, University of Debrecen. Reads were aligned to the Human Genome (v 19) using Map with Bowtie for Illumina. Enriched gene ontology terms (GO) were derived using PANTHER Classification System.

RNA isolation

Total RNA was extracted using an RNA extraction kit according to the manufacture's protocol. Briefly, cells were lysed using TRIzol reagent and the homogenized samples were transferred into the Zimo Speen column. After centrifugation and DNAase I digestion, the RNA was washed several times with washing buffers (provided in the kit) and eluted in clean RNAase-free Eppendorf tubes.

Reverse transcription and real-time quantitative PCR (RT-qPCR)

The cDNA preparation was assessed by a High capacity cDNA reverse transcription kit. 1 µg from the total mRNA was used for reverse transcription. Real-time qPCR was performed using SYBR Premix Ex Taq II, according to the manufacturer's protocol. RT-q-PCR was performed with a LightCycler 480 Thermocycler (Roche) instrument. Data were normalized to *GAPDH* and/or *β-ACTIN* housekeeping genes. $2^{-\Delta\Delta Ct}$ values were used to calculate the fold change in the mRNA expression levels.

Extracellular flux analysis

3.5×10^4 PA200 down-regulated cells/well and the respective control cells were seeded in an XF96 cell culture microplate. To measure the oxygen consumption rate (OCR), the growth medium was replaced by a 180µl XF assay medium supplemented with 4.5 g/l glucose. To quantify the extracellular acidification rate (ECAR), the growth medium was replaced by a 180µl XF assay medium without glucose. Before the measurement, the plates were incubated at 37°C without CO_2 for 1 hour. The mitochondrial inhibitors used in the measurement of OCR were used at the following final concentrations: 1.5 µM oligomycin, 1 µM FCCP, and 1 µM antimycin-A/rotenone. For ECAR measurement, the drugs were used at the following final concentrations: 10 mM glucose (Glu), 1 µM oligomycin (Olig), 50 mM 2-deoxy-D-glucose (2-DG). The measurements were carried out in Seahorse XF96 Analyzer. Bradford assay was used to measure protein concentration for normalization. Data analysis was performed by Wave 2.3 Agilent Seahorse Desktop software.

Flow cytometry analysis

Cell cycle analysis, mitochondrial membrane potential ($\Delta\Psi m$), and ROS production measurements were assessed using Propidium iodide (PI), tetramethylrhodamine ethyl ester (TMRE), and deep red ROS detection dye, respectively. Data acquisition was performed using a FACS Aria III flow cytometer and analyzed with FlowJo software version 10.

RNA sequencing (RNA-seq)

PA200 depleted and control cells were seeded in T-75 flasks in DMEM high-glucose to reach 90% confluency. The total RNA was extracted in normal condition according to the protocol described above (see RNA extraction). The samples for RNA sequencing were performed on the Illumina sequencing platform, Center for Clinical Genomics and Personalized Medicine, Core Facility, University of Debrecen. Raw sequencing data (fastq) was aligned to human reference genome version GRCh38 using HISAT2 algorithm and BAM files were generated. Downstream analysis was performed using StrandNGS software (www.strand-ngs.com). BAM files were imported into the software. DESeq1 algorithm was used for normalization. To identify differentially expressed genes between conditions, Moderated t-test with Benjamini-Hochberg FDR for multiple testing corrections was used.

Mitochondrial morphology analysis using High Content Screening (HCS) system

PA200-deficient and control cells were seeded at the density of 1.5 x 10^4 cell/well in cell carrier-96 ultra microplates in DMEM high-glucose. Cells were treated for 24h either with DMSO 1% (v/v) or 3 µM oligomycin, then incubated at 37°C in a 5% CO_2 incubator with 50 nM Mitotracker Red CMXRos and 10 µM Hoechst 33342 in serum-free media for 30 minutes. Live microscopy image acquisition was assessed in Opera Phenix High-Content Screening System (63 x, NA=1.15). Images were analyzed using Harmony 4.8 software.

Protein carbonyl assay

The detection and quantification of proteins modified by oxygen free radicals were assessed using OxyBlot™ Protein Oxidation Detection Kit. Briefly, PA200-deficient cells and the corresponding control were seeded in 6 well plates in DMEM high glucose. The following day, cells were treated with DMSO and 3 µM oligomycin for 24 h. Cells were lysed by RIPA buffer supplemented with a protease inhibitor cocktail and 50mM DTT for 5 min at 4°C. Two aliquots of each sample containing 20 µg protein / each were treated as the following; first, the proteins of both aliquots were denatured with 12% SDS (v/v) (6% SDS final) for 5 min at RT. One of the samples was derivatized with the derivatization reactions 1x DNPH solution, while the other aliquot serves as a negative control. The protein samples were separated by SDS-PAGE. Nonspecific sites were blocked by blocking/dilution buffer, 1% BSA in PBS-T (PBS-T: PBS containing 0.05% Tween-20, pH 7.2-7.5) for 1 hour with gentle shaking at RT. The primary antibody was diluted 1:150 in blocking/dilution buffer and applied overnight. The secondary antibody was applied for 1h at RT. Images were taken using a ChemiDoc Imager. The image adjustment and quantification of bands were assessed using Image Lab software

Statistical analysis

Data from each experiment were summarized with the mean and standard deviation (SD) of $n \geq 3$ experiments. Statistical analysis was performed with ANOVA or unpaired Student's *t*-test using GraphPad Prism v8.2.1. Statistical significance was determined as * $p<0.05$, ** $p<0.01$, ***$p<0.001$, ****$p<0.0001$.

RESULTS AND CONCLUSIONS

Loss of PA200 sensitizes cells to rotenone, but not oligomycin-induced cell death

We investigated the role of PA200 on cell viability using the SRB assay. PA200-deficient cells and corresponding control were subjected to mitochondrial inhibitors for 24h. Treatment with either 100 nM antimycin A or 3µM oligomycin did not show significant changes in cell viability between the two clones. 10µM rotenone treatment, however, significantly reduced cell viability of PA200 depleted cells compared to control cells. The impact of rotenone on cell viability in shPA200 cells was confirmed by investigating necrotic and apoptotic markers. As expected, treatment with rotenone significantly increased LDH release and apoptotic markers, such as H2AX phosphorylation at serin139 and cleaved PARP 1 in shPA200 cells, compared to rotenone-treated control cells. Oligomycin treatment did not show significant changes in LDH release and apoptotic markers in shPA200 cells, compared to control cells. SH-SY5Y cells treated with rotenone are considered as an *in vitro* model for Parkinson's diseases (PD). In our study, depletion of PA200 in SH-SY5Y and treatment with rotenone led to significant massive cell death compared to rotenone-treated control cells. This data suggested that PA200 has a crucial role in cell survival and preventing neuronal disorders

PA200 knockdown leads to reduced c-Jun following rotenone administration

One known pathway for rotenone-induced cell death is through the activation of the JNK pathway and by phosphorylation of c-Jun in SH-SY5Y cells. Rotenone treatment shows significant changes in cell viability and apoptosis in PA200-deficient cells compared to control cells. We tested the phosphokinase profile of both clones upon rotenone treatment using a human phosphokinase array. c-Jun phosphorylation upon rotenone treatment was significantly higher in control cells compared to PA200-deficient cells. These findings were confirmed by western blot analysis which shows that upon rotenone treatment the level of c-Jun and phospho-c-Jun were significantly less in shPA200 cells compared to control cells. Notably, total JNK phosphorylation was similar in both clones. Thereby, reduction of c-Jun and phospho-c-Jun in shPA200 cells upon rotenone treatment was not due to less activity of JNK but, from the diminution of protein level of c-Jun (c-Jun pool) as shown in the gene expression level and western-blot analysis. Low expression of c-Jun and phospho-c-Jun in shPA200 cells upon rotenone treatment reduces the tolerance of the extracellular

stimuli (rotenone), thereby the apoptosis occurs with a high rate in PA200-deficient cells. Together this data suggested that PA200 influences cell survival at the transcriptional level through the c-Jun pathway.

PA200 is a chromatin component in SH-SY5Y neuroblastoma cells

Studies demonstrated that the deletion of PA200 orthologue in yeast (Blm10) shows an overall change in gene expression encoding for proteins required for proper chromosome organization, assembly, function, repair, and cell cycle. Based on differential sensitization of PA200-deficient and control cells to mitochondrial inhibitors and the impact of Blm10 deletion on the gene expression, we hypothesized that the depletion of PA200 in SH-SY5Y cells is associated with overall changes in the gene expression through the direct or indirect binding (as co-factor) of PA200 on relevant genes involved in mitochondrial integrity, cell stability, and survival. We carried out ChIP followed by ChIP-seq analysis in the presence or absence of mitochondrial inhibitors in SH-SY5Y cells. Gene ontology annotation has showed that PA200 is recruited to promoter regions of genes involved in cell cycle progression, protein modifications, and metabolism. Distribution of protein peaks revealed that PA200 was centered in the vicinity of transcription start sites (TSS). Furthermore, mitochondrial inhibitors lead to the eviction of PA200 from one promoter region to another. This data suggested that mitochondrial status defines the association and distribution of PA200 in the genome. Furthermore, flow cytometry and protein data analysis revealed that oligomycin-treated shPA200 cells show a significant increase in the S phase population compared to vehicle-treated shPA200 cells. This data suggested that the cell cycle is shifted in PA200-depleted cells in a mitochondrial inhibitor-dependent manner.

The effect of PA200 on the global transcriptomic profile of SH-SY5Y cells

Our ChIP-seq data revealed that PA200 is recruited to the promoter region of genes involved in primary metabolic processes. However, these findings needed confirmation by functional assays to validate the role of PA200 in metabolism. Therefore, we performed RNA-seq analysis upon stable silencing of PA200 in SH-SY5Y cells and its respective control. RNA-seq data analysis was strongly in accordance with ChIP-seq data findings. The RNA-seq revealed that loss of PA200 leads to an overall transcriptomic change in genes related to apoptosis, metabolism, cell cycle, MAPK signaling pathway, and mitochondrial homeostasis. Furthermore, the intervention of

PA200 in metabolism appears in mitochondrial homeostasis, oxidative phosphorylation regulation, ATP production, and glycolysis. In support of the role of PA200 in metabolism and the maintenance of mitochondrial homeostasis, yeast deleted of Blm10 exhibits mitochondrial dysfunction showing a reduction of respiration. Furthermore, this strain is unable to grow on non-fermentable carbon sources, such as glycerol. Moreover, the expression level of Blm10 increased upon the switch from fermentation to oxidative metabolism. In summary, our data suggested that oxidative phosphorylation might be interrupted by the loss of PA200. These observations in yeast and the results of DEG clustering of RNA-seq data pointed us to investigate the metabolic state of cells stably depleted of PA200.

Mitochondrial stress assay indicates mitochondrial dysfunction in shPA200 cells

We have validated DEGs affected by the depletion of PA200 and shown to be involved in metabolism and mitochondrial homeostasis. Seahorse data analysis revealed that PA200 cells exhibit defects in mitochondrial activity. Lacking PA200 leads to a significant reduction in the spare respiratory capacity, maximal respiration, and proton leak. Reduction in spare respiratory capacity and maximal respiration indicates that mitochondria are not able to generate enough energy by oxidative phosphorylation to overcome stress. This data show that loss of PA200 negatively affects oxidative phosphorylation, especially under conditions where cells are in high energy demand. Furthermore, western blot analysis of mitochondrial oxidative phosphorylation complexes in PA200-deficient and control cells show a significant reduction of ETC complexes in shPA200 compared to control cells, especially the CI subunit, NDUFB8, which is involved in CI assembly. The level of the ATP synthase subunit, ATP5E did not show changes between the two clones, which might explain the similar OCR rate in baseline and ATP production in both clones. This data suggests that PA200 affects mitochondrial biogenesis and that leads to inefficient mitochondrial function under conditions where cells need high energy.

PA200 knock-down cells shift from OXPHOS to glycolysis

As we described above, mitochondrial stress assay indicates mitochondrial dysfunction in shPA200 cells. This raised the question how PA200-deficient cells adapt to mitochondrial dysfunction? The answer primarily appeared in the RNA-seq data. DEGs show that PA200 is a repressor of glycolysis and an activator of negative regulation of glycolysis. As we expected, the

glycolytic stress assay revealed that loss of PA200 leads to a significant increase in glycolysis and glycolytic capacity. These data suggest that the interrupted OXPHOS activity in cells stably lacking PA200 drives cells to alternative sources of energy, such as glycolysis, to produce more ATP and adapt to the inefficiency of mitochondrial function.

Depletion of PA200 leads to alteration of mitochondrial structure

The mitochondrial network structure correlates with the energy status of the cell. We speculated that the impaired mitochondrial activity in PA200-deficient cells has an impact on mitochondrial morphology. Mitochondrial morphology is tightly regulated by proteins with GTPase activity. We investigated mitochondrial morphology of PA200-deficient and control cells with Mitotracker Red CMXRos dye using the Opera Phenix HCS system. HCS data analysis revealed that both shPA200 and control cells under physiological conditions did not show any morphological differences in mitochondrial classes, however, when cells were challenged with oligomycin, shPA200 cells exhibited significant increases in long and compact tubular structures. Western blot analysis of key mitochondrial fission and fusion proteins revealed a significant reduction of stress-induced L-OPA1 cleavage in shPA200 cells compared to control cells. Furthermore, under the same condition OMA1 protease that cleaves L-OPA to S-OPA1 was significantly low in PA200-deficient cells upon oligomycin treatment. This data suggest that the low level of OMA1protein in shPA200 upon selective mitochondrial stress maintains L-OPA1 isoform and promotes mitochondrial fusion. Our data are also confirmed by a study describing increased glycolysis in mesenchymal cells, which promotes cell survival via Opa1-mediated mitochondrial fusion, regulated by leptin. Another report also demonstrated that the purposes of mitochondrial fusion are to ensure exchange of metabolites, energy restoration, and preventing apoptosis. Overall, our data suggest that deficiency of PA200 leads to an overall change in the transcriptomic process of relevant genes including metabolism and mitochondrial biogenesis, and homeostasis. Inefficient mitochondrial function in shPA200 cells was counterbalanced by increased glycolysis and elevated mitochondrial fusion upon stress.

Loss of PA200 increases ROS production and oxidized proteins upon mitochondrial stress

During oxidative phosphorylation, ROS are naturally generated by the mitochondria mainly by CI and CIII of the ETC. The level of ROS is adjusted by an antioxidant mechanism to prevent

mitochondrial protein damage and loss of their function. Flow cytometry data show that loss of PA200 under normal conditions significantly reduces ROS production compared to control cells. However, when cells are treated with oligomycin, ROS are significantly increased in shPA200 compared to vehicle-treated cells, while the level of ROS in control cells slightly increased compared to vehicle-treated cells. Low ROS in shPA200 cells under normal conditions might be a result of the reduction of mitochondrial ETC biogenesis, especially the assembly subunit NDUFB8 that contributes directly to oligomerization of CI. Reports demonstrated that the decrease in CI activity leads to an increase in ROS production. On the contrary, inhibition of CI assembly by repressing one of the assembly subunits leads to a reduction in ROS production. Furthermore, studies demonstrated that treatment of cardiovascular diseases with lovastatin indirectly reduces the protein level of PA200 which is accompanied by minimizing ROS-induced damage in endothelial cells. Significant increases in ROS production in PA200 depleted cells, but not in control cells upon oligomycin treatment are indicative of more damaged mitochondria in shPA200 cells.

At physiological conditions, the mitochondrial ETC complexes are exposed to ROS-induced ETC protein carbonylation and progressive mitochondrial dysfunction during normal aging. We measured low ROS production in shPA200 cells under normal conditions. Oxyblot assay revealed significantly low protein oxidation in shPA200 cells compared to control cells. Protein oxidation in PA200-depleted cells treated with oligomycin was higher compared to oligomycin-treated control cells. These data are consistent with ROS status of both cells under the indicated conditions. Together, these findings suggest that loss of PA200 negatively influences ROS production and protein oxidation as an adaptive mechanism of mitochondrial dysfunction preventing ROS-induced ETC oxidation. However, when shPA200 cells are challenged with selective mitochondrial inhibitors, cells were not able to control ROS-induced protein oxidation. One of the explanations might be the interrupted ETC biogenesis in PA200-depleted cells since ETC is the main source of ROS generation. In summary, our findings support a model in which PA200 potentially regulates cellular homeostasis at the transcriptional level and suggest a role for PA200 in the regulation of metabolic changes in response to selective inhibition of ATP synthase in an *in vitro* cellular model.

SUMMARY

The conserved Blm10/PA200 family belongs to the proteasome activators. PA200 alternatively binds to the proteasome core particle (CP) and facilitates peptide degradation in an ATP and ubiquitin-independent manner. PA200 has several important physiological roles, such as DNA repair, spermatogenesis, and aging. However, the exact molecular mechanisms behind these functions are not clear. In the present study, we investigated new promising functions of PA200 and the consequences of PA200 stable depletion at the normal condition and upon mitochondrial stress. First, we investigated the role of PA200 on cell viability and apoptosis with or without mitochondrial stress. We have found that PA200 deficient cells are more sensitive to rotenone but not oligomycin and antimycin A treatment. Furthermore, PA200-deficient cells exhibit low expression of c-Jun upon rotenone treatment, thereby; reducing the capacity of PA200 depleted cells to tolerate the rotenone-induced cell death. Second, we performed ChIP followed by ChIP-sequencing analysis to study the possible role of PA200 as a transcription factor or cofactor that regulates the transcription machinery. We have demonstrated that PA200 is a chromatin component in SH-SY5Y neuroblastoma cells and it binds to promoter regions of genes involved in the cell cycle, metabolism, and protein modification. Third, we performed an RNA sequencing study. We found that the loss of PA200 has an overall change in the transcriptomic profile of the SH-SY5Y cell line. Functional annotation analysis of differentially expressed genes (DEGs) revealed that PA200 has a crucial role in cellular functions including apoptosis, metabolism, cell cycle, MAPK signaling pathway, and mitochondrial homeostasis. Furthermore, the extracellular flux analysis of shPA200 and control cells indicates mitochondrial dysfunction and a significant increase in glycolysis in PA200 deficient cells. Further investigations of mitochondrial mass show a significant reduction in mitochondrial ETC biogenesis upon loss of PA200. Finally, mitochondrial morphology analysis shows significant increase in fused mitochondria in shPA200 cells, compared to control cells upon oligomycin treatment due to maintenance of L-OPA1 isoforms. Our new findings provide more information regarding the role of PA200 in eukaryotic cells. The new disclosed functions of PA200 in the maintenance of mitochondrial integrity and cell survival may contribute to the development of new therapy for many diseases related to mitochondrial dysfunction including but not limited to neurodegenerative disease, cardiovascular disease, and aging.

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Registry number: DEENK/102/2021.PL
Subject: PhD Publication List

Candidate: Abdennour Douida
Doctoral School: Doctoral School of Molecular Medicine

List of publications related to the dissertation

1. **Douida, A.**, Batista, F., Botó, P., Regdon, Z., Robaszkiewicz, A., Tar, K.: Cells Lacking PA200 Adapt to Mitochondrial Dysfunction by Enhancing Glycolysis via Distinct Opa1 Processing. *Int. J. Mol. Sci. 22* (4), 1-22, 2021.
   DOI: http://dx.doi.org/10.3390/ijms22041629
   IF: 4.556 (2019)
2. **Douida, A.**, Batista, F., Robaszkiewicz, A., Botó, P., Aladdin, A., Szenykiv, M., Czinege, R., Virág, L., Tar, K.: The proteasome activator PA200 regulates expression of genes involved in cell survival upon selective mitochondrial inhibition in neuroblastoma cells. *J. Cell. Mol. Med. 24* (12), 6716-6730, 2020.
   DOI: http://dx.doi.org/10.1111/jcmm.15323
   IF: 4.486 (2019)

Total IF of journals (all publications): 9,042
Total IF of journals (publications related to the dissertation): 9,042

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.

17 March, 2021

DEBRECENI EGYETEM * Egyetemi és Nemzeti Könyvtár *