STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LX, Sp. Iss., 2015 (p. 19-27)

Western Blot analysis of detoxifying enzymes, cytochrome c and sirtuin expression in patients suffering from mitochondrial complex I deficiency

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SUMMARY. In recent years, a great deal of interest has been given to sirtuins and their role in mitochondrial metabolism, especially their implication in the process of aging. By activating detoxifying enzymes such as manganese-dependent superoxide dismutase (MnSOD) or copper-zinc superoxide dismutase (CuZnSOD), sirtuins are able to trigger enzymatic cascades leading to the reduction of cellular reactive oxygen species (ROS). This is of high importance as ROS is one of the main factors implicated in cellular aging.

In addition, it has been recently shown that mitochondrial complex I activity influences the expression and function of sirtuins. Complex I or the NADH dehydrogenase is the largest complex of the mitochondrial respiratory chain and a major regulator of energy production. Also, it is the main site for ROS production. That being said, it is important to determine not only the exact connection between sirtuins and ROS production, but also the way complex I affects sirtuin expression and activation.

In our study we analyzed selected cell lines carrying mitochondrial DNA mutations in genes encoding for complex I subunits. Our main goal was to identify potential correlations between sirtuin expression, ROS production and specific mitochondrial DNA mutations. This has been done by measuring antioxidant enzymes and sirtuin expression using Western Blot technique. The results demonstrate that complex I deficiencies do have an impact on ROS production and sirtuin expression. Moreover, we identified a particular correlation between SIRT3 and MnSOD.

Keywords: Mitochondria, oxidative stress, sirtuins

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Introduction

To maintain its physiological parameters, as well as for various actions performed in everyday life, the human body needs energy. Since the very first eukaryotic cell, energy has been provided by sub-cellular structures known as mitochondria.

The main function of mitochondria is mostly energy (ATP) production. Apart from this, it is also involved in reactive oxygen species (ROS) production, Ca^{2+} homeostasis and cellular apoptosis. These processes depend on the structural and functional integrity of the mitochondrial respiratory chain, also known as the oxidative phosphorylation system (OXPHOS). OXPHOS is made up of five multi-subunit enzymatic complexes, expressed from both nuclear and mitochondrial genes. The five complexes use reduced coenzymes and molecular oxygen to produce cellular energy. Mitochondrial OXPHOS activity is dependent on the NAD^+/NADH ratio that is crucial for the efficiency of the mitochondrial metabolism (Alberts *et al.*, 2014).

ROS production is a side effect of energy production, an increase in its levels leading to important cellular damage. Consequently, cells possess detoxification enzymes, such as manganese-dependent superoxide dismutase (MnSOD or SOD2) or Cu-Zn superoxide dismutase (CuZnSOD or SOD1) to protect against toxic levels of ROS. Also, c-type cytochromes, components of the electron transport chain (ETC), act as ROS scavengers. Cytochrome c levels may vary in a ROS-dependent manner (Atlante *et al.*, 2000).

The main producer of ROS in the mitochondria is complex I of the ETC, the largest and the most evolved enzymatic complex of the respiratory chain. Among its 45 subunits, only 14 are crucial for the catalytic function. Half of those crucial subunits are nuclear-encoded (NDUFV1, NDUFV2, NDUFS1, NDUFS2, NDUFS3, NDUFS7 and NDUFS8) whereas the other half results from the expression of the mitochondrial genome (ND1, ND2, ND3, ND4, ND4L, ND5 and ND6) (Fassone and Rahman, 2012). Due to its important role, complex I has to be well regulated. Apart from transcriptional and translational regulation, there is also post-translational regulation, which involves phosphorylation and especially acetylation. Indeed 20% of mitochondrial proteins own an acetylation site, NDUFA9 one of the complex I subunits being one of them (Ahn *et al.*, 2008). Acetylation is dependent on sirtuin activity.

Sirtuins belong to the class III histone deacetylase family with NAD^+-dependent protein lysine deacetylase activity, which has been shown to play an important role in many physiological processes. These enzymes are characterized by highly conserved NAD^+ binding and catalytic domain (Haigis and Sinclair, 2010; Li *et al,* 2015). Therefore, it is clear that sirtuins also require a balanced NAD^+/NADH ratio, for their activation, thus being dependent on the metabolic state of the cell. Their function as deacetylases allows to activate or inactivate target proteins. One of the most intriguing roles of sirtuins is related to the regulation of cellular ROS level (Pahirar *et al.*, 2014). By activating detoxifying enzymes such as MnSOD or CuZnSOD, sirtuins are able to trigger enzymatic cascades, which lead to the reduction of cellular reactive oxygen species (ROS).

Nowadays seven mammalian sirtuins are known (SIRT1-7). Their localization is dependent on tissue or cell specificity as well as physiological conditions: SIRT1, 6, 7 are located mainly in the nucleus, SIRT1 and SIRT2 in the cytosol, and SIRT3, 4 and 5 in the mitochondria (Verdin *et al.*, 2010). This study focuses more on the characterization of SIRT3 expression in skin fibroblasts from patients carrying mitochondrial DNA mutations. SIRT3 has been chosen owing to its localization in the mitochondria.

We perfomed Western Blotting in order to determine whether there is a connection between the expression of SIRT3, MnSOD, CuZnSOD and cytochrome c, as well as the implications that mitochondrial mutations have on their levels.

Materials and methods

Sample preparation. Fibroblasts were obtained from skin biopsies taken after acquiring informed consent from seven patients with mitochondrial complex I deficiencies. Four samples with four diferent mitochondrial mutations encoding complex I subunits (Table 1) were selected for further Western Blotting analysis. As control, healthy fibroblasts obtained also from skin biopsies were used.

Table 1.

Biological material used for Western Blotting: samples from four patients with diferent mitochondrial mutations and two healthy controls

Complex I subunits	Mutation	Codes used in this study	Clinical phenotype
ND4	11778	ND4 11778 DCF	Only visual impairment
ND6	14487	ND6 14487 BM	Severe
ND1	3460	ND1 3460 BM	Mild
	3709	ND1 3697 BL	Severe
WT1, WT2	Healthy fibroblasts / controls (wild type)		

Fibroblasts were cultivated at 37 °C (5% CO_2) on DMEM-12 AmnioMAX media (Institut de Biotechnologies Jacques Boy) supplemented with 10% Fetal Bovine Serum Standard Quality (Institut de Biotechnologies Jacques Boy), 25 mg of uridine and 50 mg of pyruvate. Cells were grown in 75 cm^2 tissue culture flasks and were used after they reached 75 % confluence. To prepare the cells, two confluent flasks (about 7 x 10^6 cells/ flask) were trypsinized for 7 minutes at 37 °C (2 ml/ flask). Trypsin was inactivated with 4 ml of cell media. Cells were counted with the Z™ Series COULTER COUNTER (Beckman Coulter Inc.) and then, harvested by centrifugation (800xg, 5 minutes). The cell pellets were stored at -80 °C until further use.

Western Blot assay. Western Blotting has been used to quantify the expression of MnSOD, CuZnSOD, cytochrome c and SIRT3, based on their molecular weight.

We used Tris Sodium-dodecylsulfate (SDS) with a pH of 6.8 (1 M Tris-Base and 30 mM SDS) and one with a pH of 8.8 (300 mM Tris-Base and 30 mM SDS). Next, we made the following buffers: (i) Sample buffer: 1 M Tris-SDS with pH 6.8, 30 mM SDS, 10% Glycerol, 1% Bromophenol Blue and 5% 2β-Mercapto Ethanol; (ii) Radioimmunoprecipitation assay (RIPA) buffer (pH 8): 150 mM NaCl, 1% Triton X-100, 0.5% Sodiumdeoxycholate, 0.1% SDS and 50 mM Tris; (iii) Electrophoresis buffer (pH 8.3): 272 mM Tris-Base, 1.92 M Glycine, 35 mM SDS; (iiii) Blotting buffer (pH 8.3): 20 mM Tris-Base, 150 mM Glycine, 20% Ethanol.

Two types of running gels were prepared: (i) 8% Acrylamide gel: 12.5 ml Tris-SDS (pH 8.8), 6.65 ml Acrylamide (30%), 5.85 ml distilled water; (ii) 15% Acrylamide gel: 12.5 ml Tris-SDS (pH 8.8), 12.5 ml Acrylamide (30%). 250 µL of APS (ammonium persulfate) 10% and 12.5 µL of TEMED (Tetramethyl-ethylenediamine, Eurobio, Les Ulis, France) were added. Quickly, the gels were poured between the two plaques. Isopropanol was applied on top of them. The stacking gel (Acrylamide 3%) was prepared and 100 µL of APS and 10 µL of TEMED were then added and the mixture poured on top of the polymerized running gel (after waiting at least 30 minutes).

In order to prepare the samples, we mixed them with a 20 µl of RIPA buffer per millions of cells with antiprotease(1x CompleteTM Protease inhibitor cocktail tablets, Roche, Mannheim, Germany). They were then centrifuged (8000xg, 20 min, 4 °C) and the supernatant was recovered. Next, 30 µg of sample were diluted into an antiprotease solution (1x) in order to reach 10 µL. Eventually, samples were ½ diluted into sample buffer. This mix was then heated at 100 °C for 5 minutes.

Then, we started the electrophoretic migration on the polyacrylamide gel: the first well was filled up with a molecular weight marker (PageBlueTM Protein Staining Solution, Euromedex, Souffelweyersheim, France), while in the rest of the wells we deposited the samples. The pre-migration was started under an 100 V electric field, during 10 minutes. Next, a 150 V electric field enabled the separation of proteins over 90 min. The electrophoresis tank contained electrophoresis buffer diluted in distilled water.

Nitrocellulose membranes were cut, washed in blotting buffer and then placed on a sponge on Western Blot transfer membrane (Bio-Rad, Hercules, USA). The migration gel was carefully transferred onto the membrane after cutting off and throwing the stacking gel away. An additional sponge was used to cover the gel. The transfer was then started at 260 mA for 90 min.

For antibody incubation, the membranes were transferred in a box containing a 1:1 mixture of blocking buffer (LI-COR™) and PBS 1x and placed on a stir plate for 2 h. After that, each membrane was washed twice with PBS 1X and Tween (1%) (Sigma Aldrich, Lyon, France), for 5 minutes. The antibodies, specific for each

protein of interest, came from mouse, rabbit or goat (Table 2). We used 5 ml in each box and left it for at least 2 hours. Next the membrane was again washed twice with PBS 1x + Tween 1%. Next, 5 ml of secondary antibody were added in each box and removed 45 minutes later. The washing process was repeated. Revelation was performed according to the settings below with the Odyssey Fc imaging device by LI-COR. The bands were quantified by integration of pixel intensity and normalized to reference proteins (α-Tubulin, and VDAC) which served as an internal control. The Western Blotting analysis was repeated three times for each set of samples.

Table 2.

Antibodies and immunostaining conditions

<table>
<tr><th rowspan="2">Target</th><th rowspan="2">Supplier</th><th colspan="2">Primary antibody</th><th rowspan="2">Wavelength / Duration of image aquisition</th></tr>
<tr><th>Dilution</th><th>Species</th></tr>
<tr><td>α-Tubulin</td><td>Calbiochem</td><td rowspan="3">1/2000</td><td rowspan="3">Mouse</td><td rowspan="3">700 nm / 2 min</td></tr>
<tr><td>MnSOD</td><td rowspan="3">Abcam</td></tr>
<tr><td>Cytochrome c</td></tr>
<tr><td>CuZnSOD</td><td>1/2000</td><td rowspan="2">Rabbit</td><td rowspan="2">800 nm / 7 min</td></tr>
<tr><td>SIRT 3</td><td>Cell signaling</td><td>1/1000</td></tr>
</table>

Results and discussion

Using skin fibroblasts from patients suffering from complex I deficiency due to mitochondrial DNA mutations, we performed cell culture and Western Blot in order to assess the expression of selected proteins according to their specific roles: two detoxification enzymes (MnSOD,CuZnSOD), cytochrome c and one metabolic enzymes regulator (SIRT 3). We used α Tubulin, a cytoskeletal protein, as a reference protein to control the loading on the gel, and VDAC as a reference protein for cytochrome c, due to its role as a mitochondrial porin. A comparison between levels of expression of the same proteins from healthy fibroblasts and the ones found in patient cells harboring mitochondrial DNA mutations has been made.

The oxidative stress was assessed by quantifying the expression of the antioxidant enzymes (MnSOD and CuZnSOD) and cytochrome c which has an antioxidant role (Atlante *et al.*, 2000).

MnSOD expression compared to α-tubulin in patient cell lines harboring mitochondrial DNA mutations, showed some variations for each cell sample (Fig. 1). For example, the MnSOD band corresponding to ND1 BM presents an increased signal. After quantification, we produced a graphic that would help in interpreting the results. A slight decrease of 10% compared to the control group (WT), can be noticed for the first patient cell line which is ND1 BM. Also, both ND1 BL and ND4 DCF present a reduction of 30% and over 80% respectively. However, a significant increase of 30% can be seen for ND6 BM cell lines.

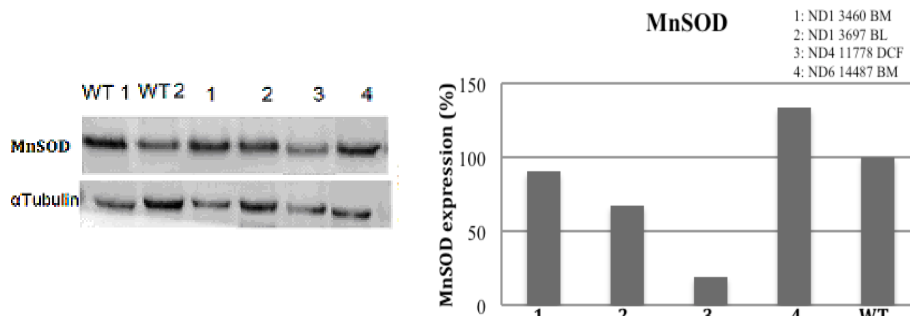

Figure 1. MnSOD levels normalized to α-tubulin in patient cell lines harboring mitochondrial DNA mutations.

For CuZnSOD, the control bands seemed to have approximately the same signal strength (Fig. 2). We noticed some differences among patient cell lines. ND1 BM presents with the lowest level, all the others being increased, or at least equal to the control. ND1 BM and ND6 BL present an increase of over 140%.

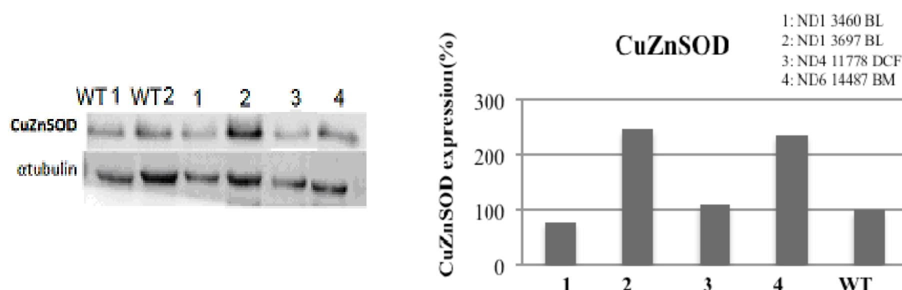

Figure 2. CuZnSOD levels normalized to α-tubulin in patient cell lines harboring mitochondrial DNA mutations.

Cytochrome c expression presented similar bands on the Western blot picture when compared to VDAC expression (Fig. 3). Cytochrome c / VDAC ratio was slightly increased in both patients carrying a mutation in the ND1 subunit (ND1 BM and ND1 BL) compared to WT. An important increase of 420% was observed for ND6 BM. ND4 DCF was almost identical to the control.

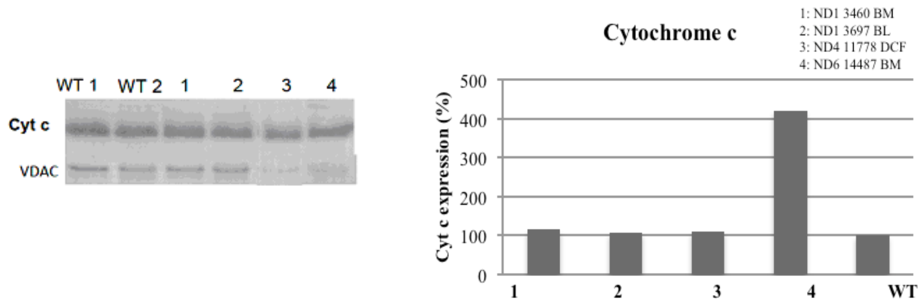

Figure 3. Cytochrome c levels normalized to VDAC in patient cell lines harboring mitochondrial DNA mutations.

We went on by quantifying SIRT3 expression in our patient cell lines. ND1 BL and ND4 DCF showed a decreased expression of SIRT3 with 20% and 30% respectively, compared to the control group. However, ND1 BM and ND6 BM had an increased level of SIRT3 expression with about 25% (Fig. 4).

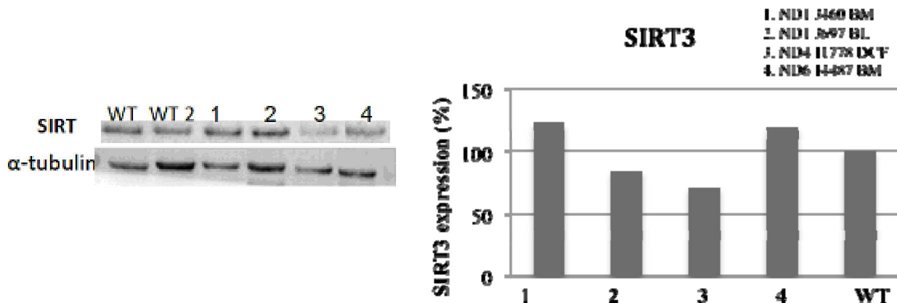

Figure 4. SIRT3 levels normalized to α-tubulin in patient cell lines harboring mitochondrial DNA mutations

Analyzing our results, SIRT3 seemed to vary on a mutation-dependent basis. Indeed, as showed on the diagram above, its expression was decreased compared to WT for ND1 3697 BL and ND4 11778 DCF whereas SIRT3 expression was induced in ND1 3460 BM and ND6 14487 BM.

In addition, a strong resemblance in the expression pattern of SIRT3 and MnSOD was observed. For each type of mutation, the variation in SIRT3 expression was similar to the change in MnSOD expression, not CuZnSOD. This observation is even more intriguing considering that both MnSOD and SIRT3 are exclusively

mitochondrial proteins, CuZnSOD being a cytoplasmic enzyme. Moreover, it is known that SIRT3 activates MnSOD by deacetylation (Bause and Haigis, 2012; McDonnell *et al.*, 2015). Lately, it has been proposed that SIRT3 would enable the induction of antioxidant genes, among which those encrypting MnSOD, through PGC-1α (Kong *et al.*, 2010). Thus, SIRT3 would not only regulate the acetylation of the protein MnSOD, but would also be able to impact its expression at a transcriptional level.

Furthermore, we observed that patients affected by a different mutation in the same subunit, displayed different biochemical profiles related to oxidative stress. For example, ND1 3460 BM and ND1 3697 BL had very different level of expression of proteins of interest such as MnSOD, CuZnSOD and Cytochrome c. The biochemical profile therefore seemed to be dependent on the location and nature of the mutation. Moreover, the biochemical profiles of patients displaying a severe phenotype are very heterogeneous, making it difficult to correlate the clinical phenotype to oxidative stress.

On the whole, the study of sirtuin regulation is an area with plenty of future possibilities, the better understanding of sirtuin function and regulation giving new insights into the process of cellular aging. Also, this knowledge would aid the discovery of new molecules acting on sirtuin expression for many illnesses, especially mitochondrial diseases, cancer and neurodegenerative diseases. Moreover, sirtuins could be the key to reducing cellular stress, thus ensuring a longer cellular life.

Conclusions

Although some major progresses have lately been made regarding the diagnosis of mitochondrial impairments, they is still no treatment available. Better understanding of the biochemical consequences of complex I deficiencies can provide a crucial support in developing new therapeutic approaches.

In our study, the oxidative stress in complex I-deficient patients was indirectly assessed through the Western blott estimation of MnSOD, CuZnSOD and cytochrome c expression. We observed that patients affected by a different mutation in the same subunit, displayed different immunoblotting profiles related to oxidative stress. Also, we noticed a possible correlation between SIRT3 and MnSOD expression in our patient cell lines carrying mitochondrial DNA mutations encoding complex I subunits.

As already mentioned, more research into this area is needed in order to be able to discover the true metabolic implications of sirtuin function.

Acknowledgements. We would like to thank the Joint Program for European Medical Studies staff for giving us the opportunity to participate in an international research program, Prof. dr. Vincent Procaccio for guiding us and sharing with us his great experience, and also Dr. Leman Geraldine for helping us throughout the entire process.

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