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Effects of mitochondrial uncoupling on Divalent Metal Transporter 1 (DMT1) isoforms

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Abstract

Humans require iron for several functions such as red blood cell production, enzyme production, and blood sugar conversion. The transferrin cycle allows iron uptake for cells to perform these vital functions. DMT1, a major metal transporter, imports iron into the cytoplasm of cells and exports it from endosomes.[3] Four DMT1 isoforms exist, with two differing in the transcription start site and two differing with the presence or absence of an iron response element (± IRE). This project focuses on these isoforms in order to begin determining their relative roles on the outer mitochondrial membrane. By uncoupling mitochondria with carbonyl cyanide m-chlorophenyl hydrazone (CCCP) to induce mitophagy and utilizing western blotting to view protein concentrations, I have compared the amount of DMT1 remaining. Current results reveal that these isoforms do not yield the same DMT1 levels after mitophagy, which raises the question of how these isoforms differ in their metabolic responses.

Introduction

Iron (Fe^{3+}) circulates in the blood stream bound to a protein known as transferrin (Tf). When Fe^{3+}-Tf reaches the cell membrane, a specific receptor protein (TfR) binds it to allow endocytosis of the complex (Fe^{3+}-Tf-TfR)[3,4]. Divalent metal transporter (DMT1) is one of the proteins responsible for the uptake of iron from the Tf cycle[6]; it has four isoforms (that differ at their beginning (N- Terminal) and the end (C- Terminal)) which are the 1A/+IRE, 1A/–IRE, 1B/+IRE, and 1B/-IRE[5] . This project utilizes Carbonyl Cyanide m-ChloroPhenyl hydrazone (CCCP – an inhibitor of oxidative phosphorylation), a poison that activates mitophagy[1], to view the relative levels of DMT1 in the cell. Doxycycline is used to induce the overexpression of DMT1 within the cells to assure a comparison between DMT1 levels. One might expect the expression of the DMT1 isoforms to decrease during this procedure consistent with their presence on the outer mitochondrial membrane (OMM).[8] Due to the presence of DMT1 on the OMM and considering how CCCP activates ubiquitin E3-ligasese during mitophagy on the OMM, some DMT1 may be turned over because of its shared location.

Results

HEK293 1A+ cell line

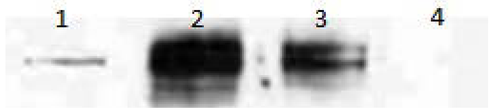

Figure 1: Western blot for 1A+ cells with 4ec DMT1 antibody. The lanes contain the following samples for all western blots: 1 control, 2 doxycycline, 3 doxycycline and CCCP, 4 CCCP.

Figure 2: Western blot for 1A+ cells with exon2 DMT1 antibody.

Figure 3: Actin bands of 1A+ cells.

Figures 1 and 2 show a decrease in the concentration of DMT1 after the addition of CCCP. Samples treated by doxycycline had more DMT1, therefore they have more visible results. Within figure 2, DMT1 was nearly absent in samples without doxycycline; therefore the comparison was done between the lanes two and three. The actin band indicates that a consistent amount of protein was loaded across the four lanes.

HEK293 2- cell line

Figure 4: Western blot for 2- cells with 4ec DMT1 antibody. The lanes contain the following samples: 1 control, 2 doxycycline, 3 doxycycline and CCCP, 4 CCCP.

Figure 5: Western blot for 2- cells with exon2 DMT1 antibody.

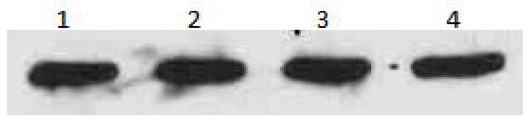

Figure 6: Actin bands of 2- cells

DMT1 in samples without doxycycline in figures 4 and 5 was undetectable. Comparing the samples with doxycycline (lanes two and three), one can see the amount of DMT1 to be slightly less in samples containing CCCP. The actin shown in figure 6 again demonstrates an equal amount of protein loaded in each lane.

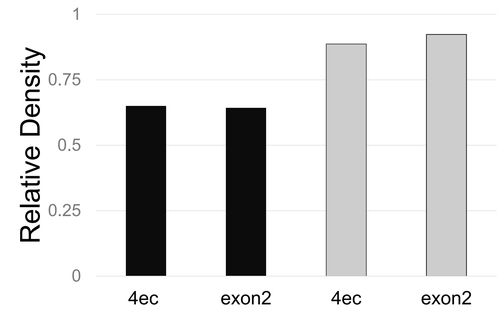

Figure 7: Graph showing relative densities of the lanes containing doxycycline and CCCP compared to lanes with only doxycycline. The black bars are the 1A + cell line and the gray bars are the 2- cell line.

Quantification of the results in figure 7 shows that the decrease in DMT1 because of CCCP treatment is greater in the 1A+ (black bars) cell line than the 2- (grey bars) cell line. Relative densities of the 1A+ sample after CCCP were 0.65 and 0.64 for 4ec and exon2 respectively while the 2- cell lines had relative densities of 0.88 and 0.92. Lanes 1 and 4 were unable to be analyzed for figure 7 due to DMT1 levels being nearly absent or undetectable.

Methods

Cell lines and cell culture: HEK 293 1A/+IRE and HEK 293 2/-IRE cells were subcultured until full confluency and then either passaged or plated for protein extraction.

CCCP/Doxycyline addition: Doxycycline was added to cell samples 48 hours prior to protein extraction while CCCP was added 2 hours prior to protein extraction.

Protein purification: Cells were lysed with a lysis buffer that contained protease inhibitors. The samples are centrifuged to separate proteins. A BCA kit is then used to determine the protein concentrations of the samples.

Western blotting: Each well is loaded with 30µg of protein. SDS-PAGE is first used to separate the proteins by mass. Nitrocellulose is then used to blot the proteins to allow for staining by antibodies. Blots were blocked with a buffer containing milk and stained with the 4ec, exon2 or actin antibody. The secondary antibody added to the 4ec and Exon2 blots was anti-rabbit IgG. ECL western blotting reagents are added to expose the blot to a film and the membrane is placed into a film developer. This film from the film developer is used view the results. Imaging software was used to quantify results from the film.

Conclusions

Both the 4ec and Exon2 antibodies are known to target DMT1. As the results from using both antibodies are similar, we have more confidence that the protein in question is DMT1, a conclusion also supported by the increase due to doxycycline. Uncoupling the mitochondria in the 1A+ cell line with the 1A/+IRE DMT1 isoform decreased it as expected, indirectly supporting DMT1's presence on the OMM. The 1B/-IRE isoform in the 2- cell line, however, yielded a noticeably smaller decrease after CCCP. Results from this cell line raise the question on how these two isoforms are different, or if their presence on the OMM differs. The Parkin E3-ligase system has been known to interact with the 1B/-IRE isoform during mitophagy.[5, 7] The current evidence also suggest that the Nedd4/Ndfip1l system is activated as well, indirectly supporting the system's role in mitophagy.[2] Between these two effector systems, the different turnover processes may be the cause for the current results. The future plan for this project is to increase the exposure to CCCP in order to verify and extend the results.

References

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