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Editorial

Investigating the Role of the Flavin Mononucleotide (FMN) Complex in Oxidative Radical Production by Targeting the Molecules Arginine (Arg) 1400, Calmodulin (CaM), and Nicotinamide Adenine Dinucleotide (NADH)

Fogel N*

Department of Neuroscience, Cell and Molecular Biology and Philosophy, University of Toronto, Canada

***Corresponding author:** Nicole Fogel, Department of Neuroscience, Cell and Molecular Biology and Philosophy, University of Toronto, Victoria College, Royal College of Surgeons in Ireland (RCSI) Medical Candidate, Canada

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Editorial

Complex I, or reduced Nicotinamide Adenine Dinucleotide (NADH): ubiquinoneoxidoreductase, is the largest enzyme of the mitochondrial respiratory chain [1]. In the first stage of the enzymatic reaction, the prosthetic group of the complex, Flavin Mononucleotide (FMN) accepts two electrons from NADH [1,2]. From here, reduced FMN passes the two electrons to a chain of eight iron-sulfur (Fe/S) centers, and ultimately to ubiquinone (CoQ). As discussed in Dr. Andreazza's lecture, there are three conditions necessary to Mitochondrial Transport Chain (MTC) production of Reactive Oxygen Species (ROS), which are "chemically reactive molecules containing oxygen" [2]. The three conditions are: "decreased production of ATP, electron carriers are fully reduced (FMN), and high NADH/NAD+ ratio in the mitochondria matrix" (caused by ischemia, for example) [2]. In order to investigate the role of the FMN complex in oxidative radical production within neurons, it would be logical to make use of these conditions, in developing an experiment.

Further, it has been implicated that in neuronal Nitric oxide synthase (nNOS), Calmodulin (CaM) binding regulates the electron flow from NADPH, through FMN, as described above, to the haemoxygenase domain (site of NO generation) [3]. CaM binding suppresses the electron flow from nNOS to molecular oxygen and "prevents the accumulation of reactive oxygen species" [3]. Without CaM, NADP(H) locks nNOS domains (mediated by Arg1400) and restricts the motion of FMN-binding domain, inhibiting electron transfer and also formation of NO [3].

If oxidative radical production is affected, or regulated, by such molecules as NADH, CaM, and Arg1400 [2,3], then in order to investigate the role of the FMN complex in oxidative radical production within neurons, it would be logical to test if this production is altered (or what the effects on production would be) when the molecules influencing the FMN complex in oxidative radical production are affected, by lowering the NADH/NAD+ ratio [2], targeting CaM, or mutating Arginine1400. Additionally, it is possible to examine the role of the FMN complex in oxidative radical production by either mutating the complex itself, or removing it, then observing the effects on oxidative radical production (increased or decreased effects).

One possible experiment could be to do a global study- lowering the NADH/NAD+ ratio, targeting CaM, mutating Arginine1400, and/or mutating or removing the FMN complex itself (or something inside the complex)-and then examining levels of nNOS or Ubiquinone (at the end of the pathway), or the levels of oxidative radical production (or whether they are produced). The question would be: How is oxidative radical production within neurons affected when molecules of the MTC (Arg, CaM, NADH, and FMN) pathway are affected, or altered, and how do these changes affect the FMN complex, in order to elucidate its role in oxidative radical production?

The hypothesis would be that altering components upstream of the pathway, like NADH, would affect the FMN complex, which would be apparent in the final products of the pathway, like in Ubiquinone (and even NO formation). Oxidative radical production levels with these alterations can be measured, alluding to the role of FMN in oxidative radical production (or its role can be inferred). Specifically, without CaM binding, or no electron transfer from NADH, the motion of the FMN-binding domain should be restricted and; therefore, with electron transfer from FMN inhibited, production of oxidative radicals should be lowered (or not present), if FMN is thought to play a role in regulating the rate of superoxide radical production [2].

To test this hypothesis, in a murine (mouse) model, the molecules CaM and NADH (once purified using chromatography techniques) could be targeted to lower their levels in the mitochondria of neurons in the prefrontal cortex (an area that seems to be involved in such studies and would be a larger area to determine effects), using an inhibitor molecule, or drug, either suggested from previous studies or screened to detect specific molecules, or drugs, for use. Certain mediators discussed in previous studies could also be added to promote one-electron transfer, instead of two-electron transfer, then titrated. By lowering the levels of (or in the absence of) CaM and NADH, the FMN complex should be negatively affected. If no electrons can be transferred to the FMN complex, production of oxidative radicals should cease, or not be present, if it can be inferred that the FMN complex regulates the production of superoxide radicals in neurons [2]. The motion of electron transfer can be measured using spectroscopy techniques; studies suggest using a Cary UV-50 Bio UV-visible scanning spectrophotometer, in combination with

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other spectroscopy techniques to ensure reliability of the results [3], or delayed red-light emission (DL spectroscopy) for estimating the "timescales of various electron transfer steps involving the formation of flavin…radicals with subsequent production of superoxide" [1].

Also, CaM or NADH variants (or supposed oxidative radical products of FMN) can be labeled with a fluorescent dye, determined by suggestions from previous studies [3], and fluorescence measurements can be performed in a fluorometer. Other methods could be used, like the EPR method for detection of free radicals (O_2- and NO production in WT versus. experimental group), or Western blotting. Fast, simple, and affordable specific assays for the detection of ROS have also been used [4] If the FMN complex is responsible for regulating the production of superoxide radicals [2], then with the upstream pathway affected, the FMN complex should not be able to produce, or form, these radicals; therefore, very few of these radicals would fluoresce under the microscope. As a final step, the data could be normalized/adjusted and analyzed using a specific software (and following its instructions), like Origin Software (Origin Lab), as outlined in a previous study [3]. Results would be compared in Wild-Type (WT) mice that do not have CaM or NADH affected (control), to the experimental group in which these molecules are affected. Downstream effects of nNOS formation and Ubiquinone production levels could also be measured using spectroscopy techniques to determine the FMN complex's role in the MTC pathway as a whole, as well. To further confirm results, Arg1400 can be substituted by another amino acid, such as Asparagine (or R1400N), which would affect mediation of CaM binding [3]. Results can be compared between WT and R1400N mutant human nNOS FMN constructs expressed and purified, using methods from previous studies.

In this study, it should be confirmed that the differences in spectra measurements between the WT and the mutant were not due to other factors, like to binding capability changes, by calculating the dissociation constant using specific measurements as previously outlined [5]. Ensuring the correct levels and specificity (detecting multiple versus single ROS) of molecular targeting and that the measurements are accurate (or are as specific as possible) would be necessary in this type of study. It should also be noted that these types of studies might not be able to simulate natural conditions *in situ* and there might be complications or limitations to conducting studies in neurons. Also, the techniques mentioned might have limitations of their own, which might affect how reliably the data can be compared among groups. The length of the study, funding amount, and alternative methods would need to be determined. Although there are confounding factors to account for, hopefully this study could provide further insights and evidence into the structure and function of the complex, specifically, the role of the Flavin Mononucleotide (FMN) complex in oxidative radical production (and regulation of the rate of production) within neurons. These insights could possibly provide better diagnostic tools and could help manage mitochondrial disorders, like bipolar disorder [1,2].

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