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Review: The Bidirectional Relationship Between Mitochondrial Respiratory Chain Inhibition and Import Machinery Malfunction in Parkinson's Disease

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Introduction

Parkinson's Disease (PD) is characterized by a loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) portion of the ventral midbrain, the region of the brain largely responsible for motor control, executive cognitive function, and emotional limbic activity (Sonne 2020). SNpc dopaminergic neurons are more vulnerable than those in other regions due to their high energy demand arising from long, highly branched, unmyelinated axons (Pissadaki & Bolam 2013). They also have a higher basal rate of oxidative phosphorylation, which has been hypothesized to further increase vulnerability in cases of PD (Pacelli et al., 2015). One of the major mitochondrial dysfunctions in PD patients is respiratory inhibition, specifically brought on by a reduction in complex I activity (Bindoff et al., 1989). Studies have found that complex I activity in the SNpc of PD patients is decreased by 40% (Schapira et al., 1990).

This bioenergetic dysfunction of mitochondria in PD has been well-studied, but new questions have begun to arise regarding the role of mitochondrial import mechanisms in PD pathogenesis. In fact, several recent studies have shown the two to be related in bidirectional fashion (Figure 1). Franco-Iborra et al., (2018) found that inhibition of complex I by MPTP led to decreased expression of both TOM and TIM complexes, and by extension a decrease in import of nuclearly-encoded proteins. TIM and TOM (translocases of the inner and outer membrane, respectively) are responsible for facilitating the movement of the 99% of mitochondrial proteins encoded by the nucleus into the mitochondria, so a decrease in their expression can lead to any number of cellular issues (Koehler 2000). To summarize, Franco-Iborra et al. found that inhibition of the mitochondrial respiratory chain via complex I obstruction led to a mitochondrial protein import machinery malfunction. On the other side, Di Maio et al., (2016) found that accumulation of α-synuclein on the outer mitochondrial membrane impedes TOM complex

function, and ultimately led to decreased mitochondrial respiration. While they did not discuss the cause of α-synuclein accumulation, another study by Neethling et. al (2018) found that PD-causing mutant LRRK2 (leucine-rich repeat kinase 2) aggregates on the outer mitochondrial membrane (OMM) around the TOM complex, and it was suggested by Lichtenberg et al., (2011) that LRRK2 aggregation on the OMM inhibits the clearance of proteasome substrates and thereby causes the accumulation of α-synuclein at the TOM complex.

This review will examine the literature to analyze the varying ways in which mitochondrial import malfunction results in respiratory inhibition, and vice versa, to cause PD pathogenesis, including direct complex I inhibition by MPTP and indirect complex I inhibition via α-synuclein and LRRK2 aggregation in the outer membrane. Understanding how these import pathways possibly play into dopaminergic cell death can help researchers discover better ways to slow and maybe even stop the onset of neurodegenerative diseases like PD.
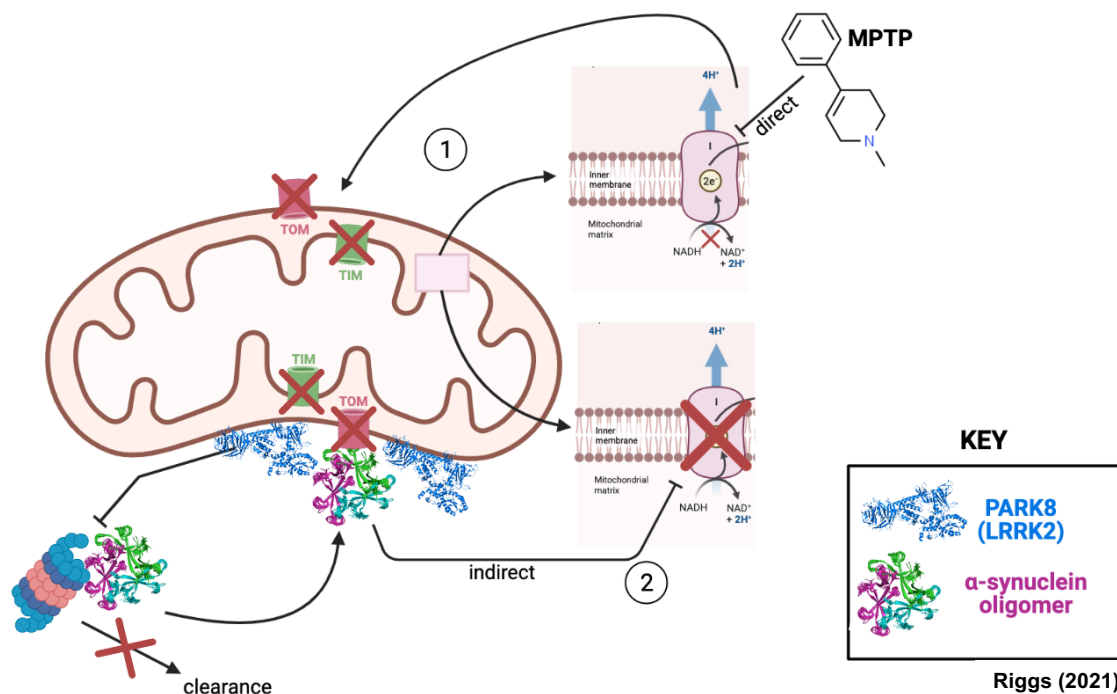
Figure 1: Theoretical schematic of bidirectional relationship between complex I inhibition and protein import machinery malfunction. (1) MPTP directly inhibits complex I by blocking the oxidation of NADH, resulting in import machinery inhibition (Franco-Ibbora 2018). (2) LRRK2 accumulates on the OMM and inhibits the clearance of proteasome substrates α-synuclein oligomeric species, which then aggregate at the TOM complex and blocks protein import, indirectly inhibiting complex I (Neethling et al. 2018, Lichtenberg et al., 2011). α-synuclein oligomer structure taken from Salveson et. al., (2016) and PARK8 from Deniston et. al., (2020). PARK8 structure pictures contain G2019S mutation mentioned in Neethling et. al., (2018). Figure rendered in BioRender.

Direct inhibition of complex I by MPTP

Direct inhibition of complex I of the electron transport chain ETC was known to be associated with dopaminergic cell death and subsequent onset of PD. Researchers knew going into this study that errors in mitochondrial protein transport into the matrix via translocation machinery (i.e. TOM20 and TIM23 complexes) could lead to various neurodegenerative diseases in humans, such as X-linked human deafness dystonia syndrome (Tranebjaerg et. al., 1995). Additionally, researchers knew that mitochondrial import can be modified in response to various stressors, such as external stimuli, oxidative stress, and aging. While this bioenergetic deficit in mitochondria is adequate to cause PD, little is known about how a failure of mitochondrial protein import mechanisms could lead to the onset of PD as well. Other neurodegenerative diseases have been found to be caused by deficient mitochondrial import machinery, and because PD is already known to be associated with the bioenergetic aspect of mitochondrial dysfunction, researchers were interested in looking into the import pathway during PD disease states to see if modification could be the cause. This then led them to their research question: is dysfunction of mitochondrial protein import associated with dopaminergic cell death, and if so, can correcting this deficiency reduce dopaminergic cell death and slow the process of PD?

This study came away with five main conclusions: (1) there is a mitochondrial protein import machinery deficit in the SNpc portion of PD brains, (2) complex I inhibition impairs the import of proteins into the mitochondria, (3) TOM20 and TIM23 overexpression can prevent cell death in vitro, (4) mitochondrial protein import machinery impairment precedes dopaminergic neuron degeneration in the MPTP mouse model of PD, and (5) TOM20 and TIM23 overexpression cannot fully prevent cell death in vivo.

The two important conclusions from this paper are 1, 2, and 5. In order to reach conclusion (1), researchers analyzed TIM23 and TOM20 expression from post-mortem PD brains via immunoblot, in addition to expression of nuclearly encoded mitochondrial proteins NDUFS3 and COXIV in the same manner. They found in both cases that these proteins were under expressed in PD brains. For (2), they analyzed mitochondrial protein import to see if MPP+ (the active metabolite of parkinsonian neurotoxin MPTP) intoxication led to the downregulation of TOM20 and TIM23 via immunoblotting and direct assay of mitochondrial protein import in isolated mitochondria. MPTP directly inhibits complex I by blocking the oxidation of NADH, so these findings indicate that mitochondrial protein import and complex I inhibition have a bidirectional relationship where mitochondrial protein import malfunction can cause complex I inhibition, or vice versa as is the case here (Cassarino 1999). To answer the latter half of their research question, Franco-Iborra et al. wanted to analyze if overexpression of TIM23 and TOM20 in vivo would attenuate dopaminergic cell death as they found it did in vitro. After injecting mice with a single unilateral stereotaxic injection of a TOM20/TIM23 adenovirus vector, they assessed (i) the mitochondrial import system by COXIV immunofluorescence and (ii) the integrity of the nigrostriatal dopaminergic system 21 days post-intoxication. They found that neither TOM20 nor TIM23 overexpression could fully attenuate complex I-induced cell death in vivo. Even though overexpression of single subunits of TOM20 and TIM23 was not enough to fight against neurodegeneration on their own, a strategy targeting several key elements in the pathway could have a major impact.

This study found that exposure to low doses of MPP+ (<5mM) over prolonged periods of time impairs mitochondrial function but does not result in immediate cell death, which corroborates a similar study done by Zhu (2012) that found low doses of MPP+ impaired

mitochondrial translation activity. Two studies by Perier in 2012 and 2013 also found that inhibition of complex I activity disrupts mitochondrial membrane potential (MP) as this study did. Loss of MP was found to cause downregulation of matrix mitochondrial protein import, which is important for two reasons: (1) Wiedmann (2017) noted that the MP is responsible for activating the channel protein itself, and (2) Truscott (2001) found that the MP exerts and electrophoretic effect on the positively charged presequences in order to drive them to the matrix side. Finally, Yano (2014) reinforces the finding of this study that mitochondrial protein import is critical for neuronal survival by noting that knockdown of TIM23 in primary cortical and striatal neurons increased cell death.

Indirect complex I inhibition: α-synuclein accumulation

Genetic and environmental factors have been associated with PD pathogenesis, however mitochondrial defects and α-synuclein accumulation are common culprits and it is though that they are somehow connected. While soluble α-synuclein is an important regulator of mitochondrial fusion and fission when interacting with mitochondrial-associated endoplasmic reticulum membranes (MAMs), the effects of α-synuclein oligomerization on the OMM were relatively unknown (Guardia et al., 2014). Additionally, researchers knew that in addition to the central pore of the TOM complex, there are more peripheral TOM20 components that are essential to proper gating of the central pore itself. However, exactly how α-synuclein interacts with the mitochondrial protein import system was unclear, as was the physical makeup of the α-synuclein toxic species. Previous studies suggested that there is a bidirectional interaction between mitochondrial dysfunction and α-synuclein aggregation: complex I inhibition led to accumulation and aggregation of α-synuclein, and increased levels of α-synuclein led to some

kind of mitochondrial respiratory dysfunction, although the exact dysfunctional respiratory element was unknown. This then led them to their research question: how does α-synuclein interact with the mitochondrial protein import system, and how does this interaction negatively affect neurons of the nigrostriatal pathway to lead to PD pathogenesis?

This paper also had five main conclusions: (1) posttransiontionally modified species of α-synuclein binds with high affinity to TOM20, preventing its interaction with its co-receptor TOM22 and impairing mitochondrial protein import, (2) impairment led to decreased mitochondrial respiration, increased production of ROS, and loss of mitochondrial membrane potential, (3) slight knockdown of endogenous α-synuclein was enough to maintain proper import in an in vivo model of PD, (4) overexpression of TOM20 in vitro rescued protein import, and (5) toxic species of α-synuclein may be trimers and tetramers.

To establish their first and arguable most important conclusion, researchers used a rotenone model of PD in rats. Rotenone is a complex I inhibitor, similar to the MPTP used in Franco-Iborra et al. (2018), that reproduces many PD-like features including the accumulation of α-synuclein in the SNpc. Using proximity ligation assays, they showed that there was a significant increase in the interaction between α-synuclein and TOM20 in the nigrostriatal dopaminergic neurons of rats treated with rotenone in comparison to the control. Additionally, they found that the amount of imported Ndufs3, a nuclear-encoded subunit of complex I, was decreased in the rotenone-treated rats as compared to the control. To determine the effects of different posttranslationally modified α-synuclein species on protein import, proximity ligation assays (PLAs) were performed, and they found that oligomeric, dopamine-modified and S129E α-synuclein had a strong signal with TOM20, while monomeric and nitrated α-synuclein did not. They then wanted to see how these different forms disturb the interaction between TOM20 and

TOM22 subunits, so they treated cells in culture with vehicle containing one of the modified

forms of α-synuclein and again measured PL signals. In the control, the PL signal between

TOM20-TOM22 was strong, but once the α-synuclein was added, the TOM20-TOM22 signal

was lost and replaced by a TOM20- α-synuclein signal. This result suggests that the binding of

α-synuclein to TOM20 prevents its association with TOM22 and is likely responsible for the

malfunction of the TOM complex as a whole that resulted in decreased protein import.

A study by Devi et.al. (2008) showed that unmodified α-synuclein is a substrate for

mitochondrial import machinery, gets imported to the matrix, and binds to / inhibits complex I.

This study has not been replicated, and Di Maio et al., (2016) contradicts it by showing that

monomeric (unmodified) α-synuclein does not interact with TOM machinery. Another study

shows that TOM complex levels are reduced when α-synuclein are overexpressed, but again

results have not been replicated (Bender et.al., 2013). A third study demonstrated that PINK1

(PTEN-induced putative kinase 1) accumulation can be minimized with sufficient mitochondrial

import (Bertolin et.al., 2013). PINK1 accumulation on the mitochondrial surface leads to

increased recruitment of parkin protein that then signals for autophagic mitochondrial clearance,

ultimately resulting in PD pathogenesis. The authors of this study hypothesized that α-synuclein

accumulation would also alter PINK1/parkin signaling due to its ability to impair mitochondrial

import, so the authors have posed this hypothesis as a future study question.

Indirect complex I inhibition: LRRK2 aggregation

Leucine-rich repeat kinase (LRRK2) is known to be important in maintaining

mitochondrial homeostasis and has been shown to associate with outer mitochondrial membrane

(OMM) proteins. While mutations in this large multi-domain protein have been associated with

PD, the exact mechanism was unknown. The pathogenesis of PD by PINK1/parkin has been well studied, and since recruitment of parkin is dependent on accumulation of PINK1 on the OMM, researchers were interested to see if accumulation of LRRK2 on the OMM led to impairment of the TOM complex. When mitochondria are healthy, PINK1 is partly imported and then cleaved and degraded once it gets inside. However, if the mitochondrial are dysfunctional, PINK1 will accumulate on the OMM at the TOM complex. This accumulation of PINK1 triggers Parkin recruitment where is ubiquitinates lots of different OMM proteins, including subunits of the TOM complex, and thereby marks the mitochondria for destruction via mitophagy. Previous studies of PINK1/Parkin suggest that PD-causing mutations of Parkin disrupt proper Parkin/TOM binding, so the authors of this study hypothesized that LRRK2 and its PD-causing mutation may associate with the TOM complex in a similar manner to Parkin.

This study came to two important conclusions: (1) LRRK2 interacts with subunits of the TOM complex, and (2) PD-causing mutant LRRK2 forms perinuclear protein aggregates co-localized to mitochondria. To come to conclusion 1, researchers used coimmunoprecipitation analysis to see to what extent, if any, LRRK2 interacts with the TOM complex under basal and/or stress-induced conditions. In both cases of basal and stress conditions, LRRK2 and mutant were shown to interact with Tom40, the central subunit of the TOM complex. To reach the second conclusion, researchers expressed G2019S in HEK293 (human embryonic kidney) cells and subsequently detected many large protein aggregates, as opposed to small amounts of protein aggregates found in wild-type cells. They then rendered micrographs which revealed dense conglomerates in close proximity with TOM complexes, suggesting that the two may interact with one another in some way.

It has previously been suggested that protein aggregation is a hallmark of many neurodegenerative disorders (Irvine 2008), and previous studies have linked LRRK2 to the impairment of protein degradation which leads to protein accumulation (Manzoni & Lewis 2017). Specifically, Lichtenberg et al., (2011) found that α-synuclein commonly accumulates at the TOM complex when LRRK2 aggregates are present. Kim et al., (2018) corroborated this finding when they reported that accumulation of proteins in transiently transfected HEK293 cells were due to G2019S-induced protein degradation impairment. The findings of Neethling et al. provide a possible basis for the α-synuclein accumulation and subsequent import machinery impairment that leads to decreased mitochondrial respiration as described in Di Maio et al., (2016).

Potential drug therapies: anti-α-synuclein and LRRK2 aggregation

It is currently believed that aggregates of specific oligomeric species of α-synuclein are the toxic species responsible for neuronal death in the case of PD rather than just mature monomeric aggregates (Winner et., al., 2011). A 2017 paper by Pujols et. al. outlined methodology for screening potential α-synuclein aggregation inhibitors using a chemical kinetics approach to allow for quantitative detection of therapeutic molecules on α-synuclein aggregation. To track the progress of aggregation in their experiments they used Thioflavin-T (Th-T), an amyloid-specific reporter, where higher levels of fluorescence were indicative of increased aggregation. Their screening found 47 compounds that could efficiently modulate the aggregation of α-synuclein, and they were further broken down into two categories: those that decrease total aggregation without altering k1 and k2 (kinetics constants or the speed of α-synuclein aggregation), and those that decrease both total aggregation and k1 and k2. It is the

latter group that is of pharmacological interest, since they seemingly delay the onset of the aggregation reaction in addition to decreasing the overall amount of aggregation.

Additionally, researchers have also spent time looking into potential LRRK2 inhibitors, since its most prevalent mutation G2019S is one of the most common causes of both familial and sporadic PD (Fell et. al., 2015). This study served to characterize the pharmacological properties of MLi-2, a highly potent selective LRRK2 inhibitor that exhibits central nervous system activity. It displayed remarkable potency in a purified LRRK2 assay in vitro which monitored the dephosphorylation of LRRK2 pSer935. Additionally, treatment of mice in vivo led to decreased LRRK2 activity in the brain following oral dosage of MLi-2 with maximal suppression (or >90% reduction in activity) at 10 mg/kg. It was noted by Atashrazm & Dzamko (2016) that LRRK2 is responsible for a variety of functions in the cell, including trafficking and cargo sorting, cellular signaling, and inflammation and innate immunity. It is therefore important for possible therapeutics to target only the aspects of LRRK2 biology that are relevant for causing PD, but this remains a major challenge as many aspects of LRRK2 biology remain unknown.

Potential drug therapies: complex-I protection

Complex I inhibition has been shown to lead to increased production of reactive oxygen species (ROS), which in turn can attack complex I amino acid residues, creating a positive feedback loop that can cause irreparable damage (Panov et. al., 2005). Previous therapeutic studies targeting complex I protection have been done regarding myocardial ischemia (Chouchani et. al., 2013, Shiva et. al., 2007). The approach for treating this hypoxic pathological condition relied on nitric oxide (NO) – mediated modification of complex I. This particular

modification favors reversible, rather than irreversible, thiol oxidation, which prevents abrupt thiol reactivation during reperfusion and thereby prevents bursts of ROS production. Essentially, this treatment is based on the introduction of mild oxidation to provide complex I protection from sporadic ROS production associated with complex I inhibition. Inorganic nitrite is a reservoir for NO, and its introduction has been shown to restore NO signaling and provide complex I protection via reversible S-nitrosylation in the brain (Raat, Shiva, & Gladwin 2009). While myocardial ischemia occurs under hypoxic conditions, explorations of nitrite-induced protective effects have been shown to work even under normoxic conditions by reducing the production of ROS and subsequently reducing the loss of complex I function (Pride et. al., 2014). Milanese et. al., (2018) investigated nitrate as a possible neuroprotective agent in cases of PD. In short, this study found that in in vitro and in vivo experiments, nitrite administration serves as a neuroprotective of SNpc dopaminergic neurons in several preclinical models of PD, including the MPTP zebrafish model, rotenone rat model, and 6-OHDA rat model.

Conclusion

Various mitochondrial molecular pathways have been implicated in the pathogenesis of PD, but the relationship between complex I inhibition and mitochondrial import machinery malfunction has been shown to be a complex and bidirectional one. Direct inhibition of complex I by parkinsonian toxin MPTP has been shown to indirectly result in import machinery malfunction, while direct inhibition of import machinery via α-synuclein accumulation possibly due to LRRK2 aggregation indirectly results in complex I dysfunction. Both of these pathways can lead to dopaminergic neuron loss in the substantia nigra pars compacta of the midbrain and ultimately result in PD. Currently, PD treatment is mostly reactionary in that patients are given

dopamine therapy to help alleviate some of their symptoms. However, this does not actually cure the issue. It rather improves patient longevity after a PD diagnosis has been given. Research in this field is moving towards drug therapies that are preventative rather than reactionary, such as the nitrite treatment to protect complex I from reactive oxygen species damage. There have also been promising studies identifying chemical chaperones that prevent α-synuclein aggregation which is one of the most common causes of PD. PD becomes more common with increasing age, and because mitochondrial function already declines with old age (Park 2018), it is very important to identify these problematic molecular pathways and create targeted therapies that will mitigate the consequences of impaired mitochondrial protein import and complex I inhibition.

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