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Targeting Pink1-Parkin-Mediated Mitophagy for Treating Liver Injury

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Abstract

Alcoholic liver disease and acetaminophen overdose are common causes of severe liver disease and liver failure in the United States for which there is no cure. Therefore, development of new therapeutic strategies for treatment of liver injury caused by acetaminophen and alcohol is needed. We demonstrated that autophagy protects against alcohol and acetaminophen-induced liver injuries by removing damaged mitochondria via mitophagy, which is a selective form of autophagy specific for degradation of damaged mitochondria. Parkin is well-known to be required for mitophagy induction in *in vitro* models, and we previously showed that the Parkin-mediated mitophagy pathway likely plays a protective role against alcohol and acetaminophen-induced liver injuries. Therefore, pharmacological upregulation of the Parkin-mediated mitophagy pathway in the liver may provide a novel and effective therapeutic option for treatment of acetaminophen and alcohol-induced liver injuries. In this review, we discuss regulation of Parkin-mediated mitophagy and possible therapeutic targets of intervention in this pathway.

Graphical Abstract

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Keywords

Autophagy; Mitophagy; Parkin; Pink1; Liver Injury

Introduction

Autophagy is a protective process that provides nutrients during starvation by degrading and recycling excess and / or damaged organelles [1]. Autophagy can be both selective and non-selective [1,2]. We demonstrated that autophagy protects against alcohol and acetaminophen (APAP)-induced liver injuries by removing damaged mitochondria by mitophagy, a selective form of autophagy specific for degradation of damaged mitochondria [3–5]. However, the mechanism for how these mitochondria were removed by mitophagy was unknown.

Parkin was shown to be required for induction of mitophagy in *in vitro* models [6]. Parkin is an E3 ubiquitin ligase encoded by the *Park2* gene and is a 465 amino acid protein [7]; it is well known to be protective in the brain because loss of Parkin leads to development of Autosomal Recessive Parkinson's disease [8].

Even though the majority of research regarding Parkin has been related to neurodegenerative diseases such as Parkinson's disease, we found that Parkin is also highly expressed in the liver in mice [9]. Therefore, we investigated the role of Parkin in mitophagy induction as a protective mechanism against alcohol and APAP-induced liver injuries, and we found that Parkin-mediated mitophagy likely protects against alcohol and APAP-induced liver injuries by removing damaged mitochondria [10,11].

APAP overdose-induced acute liver injury/failure and alcoholic liver disease (ALD) are two major liver diseases in the United States and European countries. ALD is a chronic liver disease that is characterized by hepatic steatosis, hepatocyte cell death, inflammation, fibrosis and eventual liver cirrhosis and hepatocellular carcinoma. APAP-induced liver injury is characterized by massive central lobular hepatocyte necrosis. Currently, no successful treatments for APAP-induced liver damage and ALD are available, so novel

therapeutic interventions are needed. Mitochondrial damage is well known to play a major role in both alcohol and APAP-mediated liver injuries [12,13]. Therefore, upregulation of the Pink1-Parkin-mediated mitophagy pathway may be a beneficial therapeutic target to ameliorate progression of APAP or alcohol-mediated liver injury by removing damaged mitochondria. There are several layers of regulation for the Parkin-mediated mitophagy pathway that could act as potential therapeutic targets, which are the foci of this review.

The role of Parkin and mitophagy in acetaminophen and alcohol-induced liver injuries

We investigated the role of Parkin and mitophagy in alcohol and APAP-induced liver injuries using wild-type (WT) and Parkin knockout (KO) mice. Parkin protects against alcohol-induced liver injury and steatosis via mitophagy and maintenance of mitochondrial function [10]. Parkin KO mice have more liver injury and steatosis after alcohol treatment compared to WT mice because they have more severely damaged mitochondria and decreased mitochondrial function after treatment with alcohol, which is likely due to decreased mitophagy in addition to reduction in other functions of Parkin for maintaining mitochondrial integrity [10]. Therefore, pharmacological upregulation of Parkin-induced mitophagy may be a novel and effective therapeutic target for reducing alcohol-mediated liver injury and steatosis.

Using a mouse APAP-induced liver injury model, we demonstrated that APAP treatment increases Parkin mitochondrial translocation and levels of ubiquitinated mitochondrial proteins, suggesting that Parkin-mediated mitophagy may also occur in hepatocytes after APAP exposure [11]. Using an adenovirus shRNA against Parkin to knockdown Parkin in mouse livers, we found that these mice have decreased mitophagy and are more susceptible to APAP-induced liver injury. These results suggest that Parkin-mediated mitophagy protects against APAP-induced liver injury. However, APAP-treated-Parkin KO mice revealed surprising and intriguing results. Unlike liver shRNA Parkin knockdown mice, we found that Parkin KO mice do not have exacerbated APAP-induced liver injury but instead are protected against APAP-induced liver injury compared to WT mice. Subsequent studies revealed that Parkin KO mice have decreased c-JUN N-terminal kinase (JNK) activation along with increased Myeloid cell leukemia 1 (Mcl-1) expression and hepatocyte proliferation compared to WT mice, which all contribute to their protection against APAP-induced liver injury [11]. These opposing responses to APAP overdose in Parkin KO and acute knockdown mice are likely due to the compensatory adaptive responses in Parkin KO mice. Mice with acute Parkin knockdown may lack time needed to develop adaptive responses that are present in Parkin KO mice. Indeed, these compensatory protective mechanisms present in Parkin KO mice after APAP treatment are absent in mice with acute Parkin knockdown. Thus, the decreased mitophagy in acute Parkin knockdown mice likely had a role in their increased liver injury after APAP treatment due to the lack of removal of APAP-induced damaged mitochondria compared to WT mice. Therefore, pharmacological upregulation of Parkin-induced mitophagy may help reduce APAP-induced liver injury by removing damaged mitochondria.

Overall, our animal studies suggest that targeting the Parkin-induced mitophagy pathway may be a novel therapeutic option for prevention and / or treatment of severe liver disease caused by APAP or alcohol.

Regulation of Parkin-induced mitophagy and potential therapeutic targets for treatment of APAP and alcohol-induced liver injuries

Parkin is a cytosolic E3 ubiquitin ligase that translocates to depolarized mitochondria to initiate mitochondrial degradation via mitophagy [6]. Parkin-dependent mitophagy is regulated by many factors, with one of the most important being phosphatase and tensin homolog (PTEN)-induced kinase 1 (PINK1), which acts upstream of Parkin in the mitophagy pathway. Parkin directly interacts with PINK1 [14], and overexpression of PINK1 alone can initiate translocation of Parkin to mitochondria without mitochondrial damage [15]. The specificity for PINK1's role in recruiting Parkin is further shown by ectopic expression of PINK1 on peroxisomes, which results in recruitment of Parkin and degradation of PINK1-expressing peroxisomes by autophagy [16]. PINK1 promotes Parkin-mediated mitophagy by recruiting Parkin to damaged mitochondria.

PINK1 is normally cleaved and degraded in healthy mitochondria. However, PINK1 becomes stabilized on the outer mitochondrial membrane when mitochondria are depolarized, which signals Parkin recruitment to damaged mitochondria. To induce PINK1 cleavage in healthy mitochondria, the transporter outer membrane (TOM) complex on the outer mitochondrial membrane imports PINK1 into the inner mitochondrial membrane through the transporter inner membrane (TIM) complex where it is degraded by PARL (presenilin-associated rhomboid-like protein) [17]. Cleavage by PARL removes the N-terminus of PINK1, and the truncated PINK1 protein is released into the cytosol where it is degraded by the ubiquitin proteasome system (UPS) through the N-end rule mechanism [18]. However, when mitochondria are depolarized, import of PINK1 into the inner mitochondrial membrane is blocked, so PINK1 is no longer cleaved and degraded and instead becomes stabilized on the outer mitochondrial membrane [17]. Accumulation of misfolded proteins in the mitochondria can also stabilize PINK1 on the outer mitochondrial membrane without mitochondrial depolarization. However, the mechanism for PINK1 stabilization in this model is unknown [19]. In addition to its stabilization on the outer mitochondrial membrane, PINK1 must also phosphorylate itself on Ser228 and Ser402 to recruit Parkin to mitochondria [20]. Auto-phosphorylation of PINK1's Ser228 and Ser402 residues increases PINK1-induced recruitment and phosphorylation of Parkin and ubiquitin, which is necessary for recruitment of Parkin to damaged mitochondria for induction of mitophagy [20,21].

In addition to mediating recruitment of Parkin to mitochondria, PINK1 also activates Parkin's E3 ligase activity to induce ubiquitination of mitochondrial outer membrane proteins and subsequent degradation of mitochondria in autophagosomes. PINK1-mediated phosphorylation of Parkin at Ser65 in its ubiquitin-like (UBL) domain is shown to activate Parkin's E3 ligase activity [22–24]. In addition to phosphorylating Parkin, PINK1 also phosphorylates ubiquitin at Ser65 to allow for greater recruitment and activation of Parkin. Ser65-phosphorylated ubiquitin binds to Ser65-phosphorylated Parkin with 21-fold greater

affinity compared to non-phosphorylated Parkin, resulting in greater levels of Parkin activity and mitochondrial protein ubiquitination [25,26]. PINK1-induced phosphorylation of both Parkin and ubiquitin at Ser65 acts as a feed-forward loop for ubiquitination of substrate proteins on the outer mitochondrial membrane. Once Parkin is recruited to the mitochondria and activated by Ser65 phosphorylation, PINK1 phosphorylates ubiquitin (Ser65) attached to mitochondrial proteins, which tethers Parkin to the mitochondria allowing for further Parkin recruitment and ubiquitination of outer mitochondrial membrane proteins by Parkin to initiate mitophagy [27,28]. Due to its requirement for Parkin recruitment to damaged mitochondria and activation of its E3 ligase activity, PINK1 is an optimal therapeutic target for upregulating the mitophagy pathway to help reduce alcohol and APAP-induced liver injuries. Upregulation of mitophagy was recently accomplished *in vitro* by stabilizing PINK1 on the outer mitochondrial membrane via an ATP analog, kinetin triphosphate, which recruits Parkin to the mitochondria and prevents cell death in carbonyl-cyanide m-chlorophenyl hydrazine (CCCP)-treated Parkin-expressing HeLa cells [29]. Future work is needed to further validate the protective role of kinetin triphosphate to induce PINK1-Parkin mediated mitophagy *in vivo* in APAP or alcohol models. In addition to PINK1 stabilization, activation of PINK1 kinase activity could also be a therapeutic target since PINK1 auto-phosphorylation and phosphorylation of Parkin and ubiquitin are needed for Parkin recruitment to damaged mitochondria and for activation of its E3 ligase activity. However, this avenue for regulation of Parkin activity has not yet been explored. In addition, molecules that mimic PINK1-induced phosphorylation of Parkin or small peptides that mimic Ser65 phosphorylated ubiquitin may also be potential therapeutic options for upregulation of the mitophagy pathway.

Parkin E3 ligase activity is also regulated by Parkin's conformational state. Parkin has several domains including a UBL domain, which is recognized by the proteasome, and three RING (really interesting new gene) domains (RING0, RING1, and RING2) in addition to an IBR (in between RING) domain. The RING1 domain contains an E2 ligase binding site, the RING2 domain contains a catalytic cysteine residue (Cys431) necessary for transfer of ubiquitin from Parkin to substrate, and the RING0 domain is a zinc-finger domain [30,31]. Parkin is a RING-HECT hybrid E3 ligase. To ubiquitinate a substrate protein, Parkin first transfers ubiquitin from an E2 ligase to its RING1 domain and then moves it to its RING2 domain. The ubiquitin is transferred within the RING2 domain to a catalytic Cys431 residue before being further transferred to a lysine amino group on the substrate protein [30,32–34]. Parkin, unless activated, remains in an auto-inhibited state by several mechanisms, preventing its transfer of ubiquitin to substrate. The RING0 domain blocks the E2 catalytic Cys431 residue within the RING2 domain, preventing ubiquitin transfer. A repressor element of Parkin also blocks the E2 binding site on the RING1 domain, preventing acceptance of ubiquitin from an E2 ligase. Therefore, a conformational change in Parkin is required for ubiquitin transfer to substrate [30,32,35]. Theoretically, Parkin activation and induction of mitophagy could be increased by changing its auto-inhibiting conformation to an active conformational state. PINK1 phosphorylation of Parkin likely initiates a conformational change in Parkin because it has been shown to activate its E3 ligase activity [22–24], but methods for changing the conformation of Parkin with or without targeting PINK1 have not been developed.

A genome-wide small interfering RNA (siRNA) screening led to the discovery of several key genes that negatively or positively regulate Parkin mitochondrial translocation. The outer mitochondrial membrane 7 (TOMM7) and HSPA1L (an HSP70 family protein) positively, whereas SIAH3 (a mitochondrial resident protein) and Bcl2-associated athanogene 4 (BAG4, a nucleotide exchange factor for HSP70) negatively, regulate Parkin mitochondrial translocation [36]. Using viral gene delivery approaches by overexpression of TOMM7 and HSPA1L or knockdown of SIAH3 and BAG4 may improve Pink1-Parkin-mediated mitophagy and protect against APAP or alcohol-induced liver injury. Given the well-developed and successful viral vector-mediated gene delivery to mouse livers, future studies to validate these approaches in mouse APAP or alcohol models would be very interesting.

In addition, four of Parkin's different cognate E2 co-enzymes (UBE2D, UBE2L3, UBE2N and UBE2R1) also positively or negatively regulate Parkin's activation, translocation and enzymatic functions during mitophagy [37,38]. Neuregulin receptor degradation protein 1 (Nrpd1), an E3 ubiquitin ligase, regulates the ubiquitination and proteasomal degradation of Parkin. Nrpd1 interacts with C-type lectin domain family 16, member A (Clec16a), a protein that is associated with type 1 diabetes mellitus, and deletion of Clec16a increases Parkin expression. Intriguingly, despite an increased expression of Parkin, pancreas-specific Clec16a knockout mice had abnormal mitochondria with reduced oxygen consumption and ATP concentration in pancreatic β cells due to defects of mitophagy. Subsequent studies revealed that Clec16a deficient fibroblasts had defective fusion of autophagosomes with lysosomes [39], suggesting that Clec16a also regulates autophagy in addition to Parkin-mediated mitophagy.

Once recruited to the mitochondria, Parkin ubiquitinates several mitochondrial outer membrane proteins including the mitochondrial fusion proteins Mfn1 and Mfn2, Miro, Translocase of outer mitochondrial membrane 20 (TOMM20), and Voltage-dependent anion channel (VDAC) to initiate mitophagy [40]. Parkin ubiquitinates several mitochondrial outer membrane proteins through lysine48 and lysine63 ubiquitin linkages [41] along with lysine6 and lysine11 linkages [27,42], which also play roles in regulation of the mitophagy pathway. Ubiquitination and proteasomal degradation of Mfn1 and Mfn2 results in mitochondrial fission and fragmentation [41,43–45]. Mitochondrial fission has been shown to be important for mitophagy induction in liver hepatocytes among other cell types [46,47]. Fragmented mitochondria can fuse together if they have normal membrane potential, but loss of membrane potential prevents fusion and leads to mitochondria segregation and subsequent degradation by mitophagy [47]. In contrast, excessive fusion of mitochondria has been shown to inhibit the mitophagy process [48]. These observations support the notion that mitochondrial fragmentation promotes mitophagy induction. In addition to regulating mitochondria fusion and fission, Parkin-induced ubiquitination of Miro induces mitochondrial arrest, which can segregate damaged mitochondria from healthy mitochondria prior to mitophagy [49].

In addition to regulating mitochondrial dynamics and movement, Parkin-mediated mitochondrial outer membrane protein ubiquitination can also recruit autophagy receptor proteins to damaged mitochondria. Autophagy receptor proteins are a group of proteins that

share common structure similarities, which allow them to bind both ubiquitin and LC3 (microtubule light chain 3 protein) and thus recruit LC3 positive autophagosomes to autophagy receptor-decorated mitochondria for selective mitophagy [50,51]. Several autophagy receptor proteins that can bind to mitochondria after Parkin mitochondrial translocation have been reported and include p62/SQSTM1, NBR1 (Neighbor of BRCA Gene 1), TAX1BP1 (Tax1-binding protein 1), NDP52 and optineurin [52–55]. Using TALEN and CRISPR/Cas9 gene editing to delete all 5 autophagy receptor proteins in HeLa cells, Youle's group recently reported that NDP52 and optineurin, but not p62, activates Parkin-mediated mitophagy [55]. Furthermore, phosphorylation of optineurin by TANK-binding kinase 1 (TBK1) increases the association of optineurin with LC3, which is crucial for effective mitophagy mediated by optineurin. Interestingly, they further found that phosphorylated ubiquitin at Ser65 by Pink1 has a dual role in regulating mitophagy. In addition to a previously discussed role in promoting Parkin E3 ligase activity, Pink1-phosphorylated ubiquitin can directly recruit NDP52 and optineurin to mitochondria independent of Parkin. The mitochondrial bound NDP52 and optineurin then further recruit the early autophagy signaling proteins including ULK1 (Unc-51 like autophagy activating kinase 1), DFCP1 (Double FYVE-containing protein 1) and WIPI1 (WD repeat domain phosphoinositide-interacting protein 1) to trigger mitophagy, although the level of this Pink1-mediated mitophagy induction is lower than Pink1-Parkin-mediated mitophagy. Nevertheless, these findings are very important because they provide a novel mechanistic basis for targeting Pink1-mediated mitophagy for these cells with low or undetectable Parkin expression, particularly in most cancer cells.

Substrate ubiquitination by Parkin is negatively regulated by deubiquitinating enzymes (DUBs) including USP15, USP30, and USP8. USP15 and USP30 are deubiquitinating enzymes that remove ubiquitin added to proteins by Parkin, thereby inhibiting Parkin-mediated mitophagy downstream of Parkin recruitment to mitochondria. Knockdown of both USP15 and USP30 were shown to enhance Parkin-mediated mitophagy and mitochondrial clearance in neurons and in *Drosophila* [42,56,57]. Moreover, knockdown of USP30 improves mitochondrial integrity in Parkin- or Pink1-deficient flies and protects flies against paraquat toxicity *in vivo* by ameliorating defects in dopamine levels, motor function and organismal survival [56]. In contrast, knockdown of USP8, a protein that functions to remove non-canonical K6-linked ubiquitin chains on Parkin itself, delays Parkin mitochondrial translocation and impairs Parkin-mediated mitophagy [58]. Therefore, modulation of these DUBs may be another therapeutic target for activating Parkin-mediated mitophagy. Compounds inhibiting USP8 have been developed [59] and would likely exacerbate alcohol and APAP-induced liver injuries by inhibiting Parkin translocation to damaged mitochondria. Therefore, pharmacological stabilization of USP8 or inactivation of USP15 and USP30 may be potential therapeutic targets for upregulating Parkin-induced mitophagy and in turn protect against drug-induced liver injury. However, it should be noted that the DUBs are regulated both transcriptionally and post-translationally [60], which may make drug development for targeting these specific enzymes challenging. In addition, phosphorylated ubiquitin was shown to be resistant to DUBs [61]. As previously discussed, PINK1 phosphorylates ubiquitin to further enhance Parkin recruitment and activation [25–

28]. Therefore, inhibiting DUBs may not be as effective of a target as upregulating PINK1 stabilization or kinase activity.

Parkin is also regulated both transcriptionally and post-translationally. Gene mutations can occur throughout the *Park2* gene and cause loss of Parkin function [35,62]. In addition to genetic mutations, Parkin can be inactivated post-translationally via oxidation, nitrosylation, or phosphorylation [63]. Therefore, targeting of Parkin post-translational modifications to prevent its inactivation is also a potential option for upregulating the Parkin-induced mitophagy pathway. Another possible therapeutic target would be to upregulate compensatory mitophagy mediators responsible for inducing mitophagy in the liver in the absence of Parkin [10,11]. However, these pathways are not yet defined. These pathways were discussed in detail in recent reviews [9,64]. Regulation of Parkin-induced mitophagy and potential therapeutic targets for its upregulation for treating liver tissue injury are shown in Figure 1.

Concluding Remarks and Future Perspectives

Pharmacologically upregulating the Pink1-Parkin-induced mitophagy pathway may be a potential therapeutic option for treatment of liver disease mediated by APAP or alcohol. Parkin-induced mitophagy has multiple layers of regulation that could be targeted including recruitment and activation of Parkin by PINK1, induction of a conformational change in Parkin, and positive and negative regulation of this pathway by DUBs. In addition, therapeutics targeting Parkin would have to be carefully designed due to the many roles Parkin has in maintaining cellular homeostasis and mitochondrial function in addition to mitophagy [65]. Nevertheless, pharmacological upregulation of Parkin-induced mitophagy may provide a novel and effective target for treatment of alcohol and APAP-induced liver injuries by enhancing removal of damaged mitochondria. Most of the molecular mechanisms contributing to our current understanding of Pink1-Parkin-mediated mitophagy come from cell culture studies. Therefore, more *in vivo* studies pharmacologically targeting these pathways and evaluating their effects against damaged mitochondria-mediated tissue injury are needed before we can translate these findings to the bedside.

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Abbreviations

APAP	acetaminophen
ALD	alcoholic liver disease
BAG4	Bcl2-associated athanogene 4
CCCP	carbonyl-cyanide m-chlorophenyl hydrazine

Clec16a	c-type lectin domain family 16, member A
Dfcp1	Double FYVE-containing protein 1
DUB	deubiquitinating enzyme
IBR	in-between RING
JNK	c-JUN N-terminal kinase
KO	knockout
Mcl-1	myeloid cell leukemia 1
MFN1/2	mitofusin 1 and 2
NBR1	Neighbor of BRCA Gene 1
Nrdp1	Neuregulin receptor degradation protein 1
PINK1	phosphatase and tensin homolog (PTEN)-induced kinase 1
PARL	presenilin-associated rhomboid-like protein
RING	really interesting new gene
siRNA	small-interfering RNA
Tax1BP1	Tax1-binding protein 1
TBK1	Tank-binding kinase 1
TOM20	Translocase of outer mitochondrial membrane 20
TOM	transporter outer membrane
TIM	transporter inner membrane
UPS	ubiquitin proteasome system
UBL	ubiquitin-like
ULK1	Unc-51 like autophagy activating kinase 1
VDAC	Voltage-dependent anion channel
WIPI1	WD repeat domain phosphoinositide-interacting protein 1
WT	wild-type

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Figure 1. Pink1-Parkin-induced mitophagy and possible targets of intervention for treatment of alcohol and APAP-induced liver injuries

When mitochondria are damaged / depolarized, PINK1 is stabilized on the outer mitochondrial membrane where it auto-phosphorylates Ser228 and Ser402, signaling Parkin recruitment to the mitochondria. PINK1 phosphorylates Parkin at Ser65, likely causing a conformational change in Parkin so it can attach ubiquitin (UB) onto an outer mitochondrial membrane (OMM) substrate. PINK1 also acts as an UB kinase and phosphorylates UB on Ser65, increasing binding affinity of Parkin for UB and tethering it to the mitochondria. This becomes a feed-forward loop allowing for further Parkin recruitment to damaged mitochondria and increases Parkin E3 ligase activity to further increase ubiquitination of outer mitochondrial membrane proteins. Autophagy adaptor proteins, such as NDP52 and optineurin, can bind to ubiquitinated proteins on the mitochondria, resulting in autophagosome recruitment to damaged mitochondria and their eventual degradation in the lysosome. PINK1 can also directly recruit NDP52 and optineurin to mitochondria independent of Parkin to induce a low level of mitophagy. Potential targets for intervention for activation of this pathway are denoted by large red arrows and include: stabilization of PINK1 on the outer mitochondrial membrane or activation of its kinase activity, activation of USP8 to allow for mitochondrial translocation of Parkin, and inhibition of USP15 and / or USP30 to prevent deubiquitination of Parkin substrates, and activation of TOMM7 and HSPA1L or inactivation of SIAH3 and BAG4 to promote Parkin mitochondrial translocation. Other potential therapeutic options include inducing a conformational change in Parkin to allow it to remain in its active state, creating a mimetic of PINK1 to increase phosphorylation of Parkin, or upregulating Parkin-independent mitophagy pathways.