The plant triterpenoid celastrol blocks PINK1-dependent mitophagy by disrupting PINK1’s association with the mitochondrial protein TOM20

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Running Title: Inhibition of PINK1-dependent mitophagy by celastrol

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Key words: mitophagy, PTEN-induced kinase 1 (PINK1), parkin, celastrol, translocase of outer mitochondrial membrane 20 (TOM20), TOM70, microtubule, plant terpenoid, cell signaling

ABSTRACT

A critical function of the PTEN-induced kinase 1 (PINK1)–parkin pathway is to mediate the clearing of unhealthy or damaged mitochondria via mitophagy. Loss of either PINK1 or Parkin protein expression is associated with Parkinson’s disease. Here, using a high-throughput screening approach, along with recombinant protein expression and kinase, immunoblotting, and immunofluorescence live-cell imaging assays, we report that celastrol, a pentacyclic triterpenoid isolated from extracts of the medicinal plant Tripterygium wilfordii, blocks recruitment of Parkin to mitochondria, preventing mitophagy in response to mitochondrial depolarization induced by carbonyl cyanide m-chlorophenyl hydrazone (CCCP) or to gamitrinib-induced inhibition of mitochondrial heat shock protein 90 (HSP90). Celastrol’s effect on mitophagy was independent of its known role in microtubule disruption. Instead, we show that celastrol suppresses Parkin recruitment by inactivating PINK1 and preventing it from phosphorylating Parkin and also ubiquitin. We also observed that PINK1 directly and strongly associates with TOM20, a component of the translocase of outer mitochondrial membrane (TOM) machinery and relatively weak binding to another TOM subunit, TOM70. Moreover, celastrol disrupted binding between PINK1 and TOM20 both in vitro and in vivo, but did not affect binding between TOM20 and TOM70. Using native gel analysis, we also show that celastrol disrupts PINK1 complex formation upon mitochondrial depolarization and sequesters PINK1 to high-molecular-weight protein aggregates. These results reveal that celastrol regulates the mitochondrial quality control pathway by interfering with PINK1–TOM20 binding.

Introduction

Mitochondrial dysfunction is linked to a number of neurodegenerative disorders, including Parkinson’s (PD), Alzheimer’s, and Huntington diseases (1–3). Quality control of mitochondrial function is essential for cellular energetic homeostasis. Mutations in PARK 6 and PARK2 are associated with early onset of familial PD; their respective gene products, PINK1 and Parkin, have
been shown to act in the same pathway to mediate clearance of damaged mitochondria through the process of mitophagy (3, 4). PINK1, a unique Ser/Thr protein kinase that can utilize both ATP and kinetin triphosphate (KTP) (5), is imported into mitochondria, while its substrate, Parkin, resides in the cytosol. In healthy mitochondria, translocase of the outer mitochondrial membrane machinery (TOM complex) imports PINK1 into mitochondria, where it is processed by the mitochondrial processing peptidase (MPP) and continuously degraded following cleavage by the rhomboid protease PARL. Due to this process, PINK1 protein levels are low to undetectable in cells that lack mitochondrial damage (6–8).

Mitochondrial damage is induced by agents that cause excessive production of reactive oxygen species, membrane depolarization, or accumulation of misfolded proteins. These agents disrupt PINK1 import and degradation, causing PINK1 to accumulate on the outer mitochondrial membrane (OMM) (4). The buildup of active PINK1 on the OMM leads to the phosphorylation of cytosolic Parkin and ubiquitin at Ser65, which turns on Parkin’s E3 ligase activity and causes it to translocate into the mitochondria, ultimately activating mitophagy (4). Parkin ubiquitylates a diverse array of mitochondrial and cytosolic proteins (9, 10), resulting in mitochondrial repair, clearance of mitochondria by mitophagy, or apoptosis (3, 11, 12). Mitochondrial damage sensing requires tight regulation of steady-state levels of PINK1 at the OMM. TOMM 7 (translocase of outer mitochondrial membrane 7 homolog) has been implicated in releasing PINK1 and other OMM proteins laterally through the TOM complex into the OMM (13). It has not been determined how PINK1 interacts with the TOM complex or how damage signals halt its import into the mitochondria.

Celastrol is a quinone methide triterpene derived from the root of Tripterygium Wilfordii (Thunder of God Vine), which has been used in traditional Chinese medicine for the treatment of chronic inflammation, pain, and rheumatoid arthritis (14). Celastrol has been reported to possess an impressive array of biological activities, including anti-cancer (15, 16), antioxidant (17, 18), anti-inflammatory (15, 16, 19), and anti-malarial effects (20), in addition to suppression of neurodegenerative (21) and lysosomal diseases (22). However, the molecular mechanisms by which celastrol exerts these diverse physiological effects are poorly understood, and few of its mechanistically-relevant molecular targets have been identified and validated (23, 24). Among the possible cellular targets of celastrol, the best-studied has been the Hsp90 chaperone pathway (25), which celastrol inhibits through disruption of Hsp90-Cdc37 complex (23, 26, 27) or Hsp90-p23 (28). Recently, celastrol has also been linked to beige fat biogenesis as well as protection against obesity and metabolic dysfunction via activation of an HSF1-PGC1α transcriptional axis (30).

Identifying the direct molecular targets of celastrol and the cellular pathways it affects will allow us to better understand its mechanisms of action and pleiotropic effects.

In a screen to identify small molecule modulators of PINK1 accumulation on damaged mitochondria, we discovered that celastrol can block PINK1-mediated Parkin mitochondrial recruitment and mitophagy in response to mitochondrial depolarization. We show that celastrol suppresses PINK1-mediated mitophagy by two distinct mechanisms and reduces PINK1 activity. PINK1 expression is normally elevated upon mitochondrial damage; celastrol treatment suppresses this response in a cell type-specific manner. We also demonstrate that the direct interaction between PINK1 and TOM20 is disrupted by celastrol. This finding provides further molecular insight into how celastrol disrupts the mitophagy response upon mitochondrial damage.

**Results**

**Identification of celastrol as an inhibitor of Parkin mitochondrial recruitment**

The PINK1-Parkin pathway couples mitochondrial quality surveillance to mitophagy and apoptosis. Following the loss of mitochondrial membrane potential, PINK1 facilitates Parkin recruitment to damaged mitochondria to execute either mitophagy or apoptosis. Since excessive apoptosis or defects in mitophagy may contribute to the onset of PD, understanding the molecular mechanisms of mitochondrial quality control may lead to the development of new therapeutics for PD. Screening compound libraries for small molecules that can enhance or perturb Parkin’s recruitment upon mitochondrial damage could lead to the
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identification of new protein targets that are either upstream or downstream of Parkin.

As an initial effort to develop a high throughput screening assay, we created a HeLa cell line stably expressing Venus-Parkin and RFP-Mito, a fluorescent mitochondrial marker containing a targeting signal from Smac. Henceforth, we will refer to this HeLa cell line expressing Venus-Parkin and RFP-Mito as HeLa-PM. Unstimulated cells display diffuse cytoplasmic Venus-Parkin fluorescence and punctate dispersed mitochondrial red fluorescence, but within 90 minutes of treatment with the mitochondrial depolarization chemical carbonyl cyanide m-chlorophenyl hydrazone (CCCP), a protonophore, Venus-Parkin becomes punctate and colocalizes with RFP-Mito (Figure 1A). In a cell-based high content screen, we tested 77 chemical compounds (Diversity Set III, Plate No. 4734, NCI/DTP Library) to identify modulators that either promoted or inhibited CCCP induced mitochondrial recruitment of Venus-Parkin, where images were analyzed using the Transfluor module within the MetaXpress software suite (Supplementary Figure 1A). Two hits were found to significantly abrogate CCCP-induced Venus-Parkin co-localization with RFP-Mito (Supplementary Figure 1BCD). The first candidate was ethyl violet, a histological and pathological stain. The second candidate was celastrol and was selected for follow-up studies. Celastrol blocked CCCP induced Venus-Parkin recruitment to the mitochondria with an IC50 of ~2.5 µM (Figure 1B), showing celastrol to be a relatively potent inhibitor of the PINK1-Parkin mitophagy pathway.

Microtubule depolymerization does not affect Parkin recruitment to mitochondria upon mitochondria depolarization

Mitochondrial dispersion and intracellular mobility relies on mitochondrial association with microtubules and is mediated by the Mitochondrial Motor/Adaptor Complex, which consists of Miro, Milton, KHC and Dynemin/Dynactin that couple the mitochondrial surface to microtubules (31). To determine the effects of microtubule network disruptions on Parkin recruitment to mitochondria, we used HeLa-PM cells treated with CCCP and compared the effects of celastrol to nocodazole, a well-characterized microtubule depolymerizing agent, on Parkin recruitment to mitochondria. Cells were imaged for 90 min under different treatment combinations of CCCP, celastrol, and nocodazole (Figure 2). Vehicle-treated cells show well-dispersed mitochondria and diffuse cytosolic Venus-Parkin localization (Figure 2A). In cells treated with 5 µM nocodazole only, mitochondrial morphology is altered over time, but Venus-Parkin remains diffuse in the cytosol (Figure 2B). A similar pattern is seen with cells treated with 10 µM celastrol only (Figure 2C). In contrast, cells treated with 10 µM CCCP display fragmented mitochondria and Venus-Parkin colocalizes with mitochondria as early as 30 minutes, which reaches a maximum at 90 min (Figure 2D). When cells were exposed to both CCCP and celastrol, the mitochondrial recruitment of Venus-Parkin and the mitochondrial morphology effects of CCCP treatment are not observed (Figure 2E). Additionally, the combinatorial treatment of CCCP and nocodazole elicits robust Venus-Parkin recruitment to the mitochondria (Figure 2F). Taken together, these results suggest that disruption of the microtubule network by nocodazole is insufficient to prevent CCCP induced accumulation of Venus-Parkin. As a result, these findings support the conclusion that celastrol’s ability to disrupt Parkin recruitment to the mitochondria acts independently to its known function to disrupt microtubule structure and dynamics.

Celastrol suppresses the ability of CCCP treatment to elevate PINK1 protein levels

To elucidate the molecular mechanism of celastrol-induced suppression of the PINK1-Parkin pathway, we determined which steps in Parkin activation are sensitive to celastrol inhibition. Previous studies have established that the levels of PINK1 increase upon mitochondrial depolarization (32, 33). As expected in the presence of CCCP, the PINK1 levels increase and a larger molecular weight Parkin band appears in western blotting, which corresponds to phosphorylated Parkin (Figure 3A) (11, 34). Treatment with celastrol alone or in combination with CCCP did not result in a significant accumulation of PINK1 throughout 3 hours. These observations suggest that celastrol blocks the ability of CCCP to raise PINK1 protein abundance.

We further characterized celastrol by titrating it in the presence of 20 µM CCCP and determined the IC50 value of CCCP-induced PINK1 protein accumulation to be ~2.2 µM (Figure 3B),
which closely matches celastrol’s inhibition of Parkin mitochondrial recruitment (2.5 µM) (Figure 1B). These results suggest that in HeLa cells, celastrol inhibits the increase of PINK1 protein abundance induced by mitochondrial damage.

**Celastrol dependent suppression of CCCP-induced PINK1 accumulation occurs post-transcriptionally and independent of Hsp90 inhibition**

Celastrol’s effect on PINK1 protein levels can be explained by multiple mechanisms, including celastrol affecting PINK1 transcription, PINK1 translation, PINK1 degradation, or PINK1 targeting to the mitochondrial outer membrane. To probe whether celastrol can affect these processes, we first performed real-time PCR analysis of PINK1 mRNA levels in the presence or absence of CCCP and celastrol (Supplementary Figure 2BC). We found that neither celastrol alone nor CCCP plus celastrol significantly affects PINK1 mRNA levels, suggesting that the suppression of PINK1 induction occurs post-transcriptionally. Another possible mechanism of action for celastrol could be the enhancement of PINK1 degradation. PINK1 is an unstable protein with a half-life of approximately 30 minutes in cells (CHX, Figure 3C). However, upon treatment with celastrol, the metabolic stability of PINK1 does not decrease and appears to slightly increase, suggesting that celastrol does not promote PINK1 degradation. Supporting the hypothesis that celastrol affects PINK1 translation, celastrol prevents CCCP induced PINK1 protein accumulation (Figure 3D). Since celastrol is known to be an inhibitor of Hsp90 chaperoning pathway and PINK1 has been shown to associate with Hsp90 in vitro and in vivo (35–37), we investigated whether Hsp90 plays a role in celastrol-induced PINK1 dependent mitophagy. Immunoblotting analysis displays that neither celastrol nor 17-AAG, a potent Hsp90 inhibitor (10 µM), has any effect on PINK1 expression levels within 2 hours, suggesting that suppression of PINK1 induction by CCCP is not likely due to Hsp90 inhibition (Figure 3D). As shown in Figure 2E, celastrol treatment blocks CCCP induced Parkin mitochondrial translocation, but 17-AAG does not affect PINK1-dependent Parkin recruitment in the same assay (Figure 3EF). Altogether, these data lead us to conclude that the pharmacological effect of celastrol on PINK1 is likely post-translational and dependent on the mitophagy response to CCCP treatment of cells.

**Celastrol reduces PINK1 accumulation on the mitochondrial outer membrane upon treatment with CCCP**

To directly examine the effect of celastrol on the subcellular localization of PINK1, we measured expression and subcellular localization of PINK1-EGFP in the presence of celastrol or CCCP. Without stimulation, the level of PINK1-EGFP is nearly undetectable by fluorescence microscopy, presumably due to its constitutive import and degradation in healthy mitochondria (Figure 3G) (7). Confocal imaging analysis shows that PINK1-EGFP co-localizes with mitochondrial marker protein RFP-Mito upon CCCP treatment (Figure 3G). When CCCP and celastrol were added together for 2 hours, there is hardly any accumulation of PINK1-EGFP at the mitochondria. Time-lapse live cell imaging analysis of single cells was used to further confirm the inhibitory effects of celastrol on CCCP induced PINK1-EGFP levels. The intensity of EGFP that colocalizes with the RFP-Mito signal was measured as a function of time in the presence or absence of CCCP and/or celastrol (Figure 3H).

CCCP is known to cause dissipation of mitochondrial membrane potential (Δψm). As a result, one possible mechanism by which celastrol can prevent CCCP mediated PINK1 stabilization is by restoring Δψm. To test this hypothesis, we measured Δψm using tetramethylrhodamine ethyl ester (TMRE) in HeLa cells in the presence and absence of CCCP and/or celastrol. As shown in Figure 3I, celastrol treatment alone led to partial loss of Δψm. Furthermore, celastrol is unable to restore the rapid and extreme decrease in Δψm caused by CCCP. Since both CCCP and celastrol cause depolarization of the mitochondrial membrane, this result suggests that depolarization is insufficient to trigger PINK1 accumulation on the OMM or whole cells.

**Celastrol suppresses activation of the PINK1-Parkin pathway by inhibition of HSP90**

Previous studies have shown that a mitochondrially targeted chaperone inhibitor Gamitrinitriphenylphosphonium (G-TPP) triggers the induction of mitochondrial unfolded protein response and activation of PINK1- and Parkin-
dependent mitophagy (38–40). In light of the differences in mitochondrial stress elicited by G-TPP and CCCP, we tested whether G-TPP-induced PINK1 induction also can be suppressed by celastrol. In agreement with previous findings, G-TPP exposure induces mitochondrial recruitment of Venus-Parkin, which peaks at around 4 h (Figure 4A). Co-treatment with celastrol and G-TPP abrogates Venus-Parkin recruitment to mitochondria (Figure 4A and 4B).

Since G-TPP is known to activate PINK1 (40), we measured PINK1 levels and its kinase activity toward ubiquitin by western blotting. Unlike CCCP treatment, G-TPP treatment only slightly elevates PINK1 protein abundance (Figure 4C). However, G-TPP strongly promotes PINK1 activity as judged by increased phosphorylation of Ser65 of ubiquitin (Figure 4C, lanes 2 and 3). A slow migrating band corresponding to Ser65 of Ubl domain of Parkin is also evident upon incubation with G-TPP. However, neither ubiquitin nor Parkin are phosphorylated at Ser65 when cells were treated with both celastrol and G-TPP or celastrol alone (Figure 4C, lanes 5-6 and 8-9), suggesting that celastrol prevents PINK1 activation by G-TPP. This result shows that even though both G-TPP and celastrol target HSP90, they have opposite effects on PINK1-dependent events.

**Celastrol blocks CCCP-induced mitophagy**

Previous studies have shown that prolonged treatment with CCCP induces mitophagy in a Parkin-dependent manner following Parkin recruitment to the mitochondrial outer membrane (41). Three independent assays were used to assess the effect of celastrol on mitophagy induced by CCCP. First, in the early stage of mitophagy, damaged mitochondria become clustered following CCCP treatment before their eventual elimination from treated cells (Supplementary 3A) (11, 42). As shown in Figure 5A, CCCP treatment induces mitochondrial clustering after 4 h of CCCP exposure, with Venus-Parkin concentrated on the aggregated mitochondria. Celastrol alone does not affect mitochondrial clustering but blocks CCCP induced mitochondrial clustering (Figure 5AB, Supplementary Figure 3B). Next, we measured TOM20 levels by immunoblotting in cells exposed to CCCP and/or celastrol. TOM20 becomes nearly undetectable with 12 h CCCP treatment (Figure 5C, lane 3), signifying a loss of mitochondria by mitophagy after prolonged treatment. Only a partial reduction of TOM20 is seen in cells treated with both CCCP and celastrol (Figure 5C, lane 4), suggesting that celastrol partially blunts the mitophagy induced by CCCP. A third approach to measure the effect of celastrol on CCCP-induced mitophagy is by live cell imaging with HeLa cells stably expressing CFP-LC3, Venus-Parkin, and RFP-Mito. Co-localization of CFP-LC3 and RFP-Mito was used to quantify mitophagy over time (11, 43). CCCP induces a steady increase in colocalization of CFP-LC3 and RFP-mito signals (Figure 5D). No such increase is seen with celastrol or celastrol plus CCCP for at least 4 h (Figure 5D, supplementary video). However, as displayed in the supplementary video, both cell shape and mitochondrial shape changes significantly beyond 4 h, when CCCP is combined with celastrol, making two-dimensional co-localization analysis less reliable. Nevertheless, these results suggest that celastrol restrains CCCP-induced mitophagy. To determine whether celastrol blocks CCCP-induced mitophagy by suppressing LC3 processing, we performed immunoblotting analysis with an LC3 antibody comparing LC3-I expression to LC3-II expression. As expected, CCCP promotes LC3 processing while celastrol alone does not (Figure 5E, lanes 5-8 versus 9-12). With combined treatments of celastrol and CCCP, LC3 was processed similarly to CCCP alone (Figure 5E, lane 13-16), suggesting that celastrol does not affect LC3 processing in the presence of CCCP. As a result, we conclude that celastrol’s ability to prevent CCCP induced mitophagy occurs independently of LC3 processing.

**Celastrol disrupts the PINK1 protein complex formed in response to mitochondrial depolarization**

To determine whether overexpression of PINK1 can overcome the effect of celastrol on mitophagy, we stably overexpressed human PINK1 (hPINK1) in PINK1-null mouse embryonic fibroblast cells (MEFs) that also stably express Venus-Parkin. As shown in Figure 6A, overexpressed hPINK1 is readily detectable in MEF cells, and its level increases upon treatment with CCCP. Unlike what we observed in HeLa cells (Figure 3A), hPINK1 protein abundance is increased in MEF cells by either celastrol treatment or CCCP or combined celastrol and CCCP.
treatment (Figure 6A and B). However, celastrol treatment in MEFs still blocks CCCP-induced Parkin phosphorylation and mitochondrial recruitment of Venus-Parkin (Figure 6C and D). This result suggests that celastrol perturbs the function of PINK1 independent of its suppression of CCCP-induced PINK1 stabilization.

Previous analysis of the subcellular distribution of PINK1 showed that PINK1 forms high-molecular-weight complexes of approximately 700 kDa in cellular extracts prepared from CCCP-treated HeLa cells (44). To analyze PINK1 formation in MEF cells, we stably expressed Flag-tagged human PINK1 (hPINK1-Flag) in MEF cells. Upon treatment with CCCP, celastrol, or both, hPINK1-Flag levels are elevated as expected (Figure 6E). Next, subcellular fractionation studies were conducted using centrifugation (50,000 RCF) to determine where PINK1 predominantly resides among soluble and insoluble partitioning of cell lysates. The P50 fraction contains mitochondria, membranes of the endoplasmic reticulum, and plasma, as well as insoluble macromolecular complexes. In untreated cells, the small-molecular-weight cleaved form of PINK1 was mainly shown to reside in the S50 fraction, but it was largely absent in cells treated with celastrol, CCCP or both (Figure 6F, top panel, lanes 2, 4 and 6). In contrast, the full-length PINK1 resides mostly in the P50 fraction (Figure 6F, middle panel, lane 1-6). This result suggests that both CCCP and celastrol prevent PINK1 cleavage, an event that occurs following PINK1 import into the inner membrane space of mitochondria by the protease PARL (7, 8).

To analyze PINK1 complex formation upon celastrol exposure, cells treated with vehicle, CCCP, celastrol, or both were analyzed by either native or denaturing PAGE followed by immunoblotting analysis (Figure 6G). Heterogeneous high-molecular-weight PINK1 complexes were shown to be more abundant upon CCCP treatment in native PAGE (Figure 6G, top panel, lane 2). In celastrol treated cells, the heterogeneous PINK1 complexes induced by CCCP were replaced by a more homogenous higher-molecular-weight complex that barely enters the resolving gel, indicative of large aggregates of protein (Figure 6G, top panel, lanes 4 and 6). SDS-PAGE analysis shows the amount of PINK1 in cell lysates is about the same among the treatment groups except CCCP (Figure 6G, middle panel). Taken together, these results indicate that celastrol likely perturbs PINK1 function by sequestering PINK1 in large protein aggregates away from its partners on the mitochondria.

**PINK1 interaction with TOM20 is disrupted by celastrol**

Given the importance of PINK1 in mitophagy and the cellular redistribution of PINK1 we observed in celastrol-treated MEFs, we hypothesized that celastrol may affect the interaction of PINK1 with the components of the OMM. The TOM complex has been implicated in controlling the accumulation of PINK1 on the OMM (44). Two components of this complex, TOM20 and TOM70, are known to be preprotein import receptors (45). TOM20 has been shown to be involved in the initial recognition of preproteins containing the canonical N-terminal amphipathic presequence, while TOM70 often recognizes preproteins with internal targeting sequences (45).

To test for a potential interaction between PINK1 and TOM20 or TOM70, we expressed and purified recombinant GST-tagged TOM20 and TOM70 from E.coli. Full-length human PINK1 was synthesized and labeled with 35S-Met using an in vitro coupled transcription and translation system. Recombinant TOM20, TOM70, and GST control proteins were incubated with labeled PINK1 prior to the GST pull-down and SDS-PAGE. We found while both GST-TOM20 and GST-TOM70 bind PINK1 (Figure 7A), GST-TOM20 binds PINK1 with higher affinity than GST-TOM70 (more than 20% of input PINK1 was associated with GST-TOM20 versus 10% input for GST-TOM70) (Figure 7B). In the presence of celastrol, the association between GST-TOM20 and PINK1 is significantly reduced (Figure 7A and B), but celastrol treatment only has a modest effect on PINK1-GST-TOM70 binding (Figure 7A and B). The interaction between GST and PINK1 appears to be nonspecific (Figure 6B). Additionally, inhibition of PINK1 binding to GST-TOM20 is celastrol dose-dependent (Figure 7C). The specificity of celastrol was also tested with the recombinant *Tribolium castaneum* PINK1 (TcPINK1) kinase, which has been shown previously to have robust kinase activity (34, 46). No significant inhibition of TcPINK1 kinase
activity was observed with celastrol up to 160 μM \textit{in vitro} (Figure 7C).

To further address the specificity of celastrol on disrupting protein-protein interactions, we tested whether celastrol blocks interaction of TOM20 with TOM70. Previous studies have shown that TOM20 directly binds TOM70 \textit{in vitro} (47). We confirmed this result with the recombinant GST-TOM20 and His6-TOM70 (Figure 7D, lane 10). No association can be detected between the GST control and His6-TOM70. Celastrol did not affect the interaction between TOM20 and TOM70 (Figure 7D, lane 11). Collectively, these results suggest that celastrol specifically blocks the protein-protein interaction between TOM20 and PINK1.

A previous study showed that PINK1 and Parkin direct localized translation of certain nuclear-encoded respiratory chain components (nRCC) to the outer mitochondrial membrane (48). In that study, TOM20 appeared to anchor translating nRCC mRNAs and their associated ribosomes to the OMM, where PINK1 was found to associate with TOM20 in an RNA-dependent manner (48). To determine whether PINK1 interaction with TOM20 requires RNA, we incubated purified recombinant GST-TOM20 with or without RNase treatment prior to a pull-down experiment using cell lysates. These results showed that levels of PINK1 bound to TOM20 only slightly decreased following RNase treatment (Figure 7E, lanes 1 and 2), supporting the hypothesis that TOM20 is the primary binding partner of PINK1.

The effect of celastrol on PINK1-TOM20 association was investigated further in cultured cells through a co-immunoprecipitation experiment. MEF cells stably expressing hPINK1-Flag were treated with vehicle, CCCP, celastrol, or CCCP/celastrol; cell lysates from each condition were incubated with a TOM20 antibody prior to immunocapturing and immunoblotting with a PINK1 antibody. The association between PINK1 and TOM20 was shown to be significantly enhanced by CCCP (Figure 7F, lane 1 versus 2). In cells treated with both CCCP and celastrol, PINK1-TOM20 association returns to baseline levels and is indistinguishable from the control treatment. These observations provide further support for celastrol’s ability to disrupt PINK1-TOM20 association induced by CCCP \textit{in vivo}.

**Discussion**

Here we report an unbiased chemical library screen by which we identified celastrol as an inhibitor of the PINK1-Parkin pathway. Upon investigation into the mechanism of action of celastrol, we found that celastrol suppresses elevation of PINK1 protein levels in response to mitochondrial damage by sequestering PINK1 into high-molecular-weight protein aggregates and by disrupting PINK1 interaction with TOM20. These mechanisms are independent of the known microtubule disruption pharmacological activity of celastrol. Celastrol’s ability to inhibit CCCP-dependent PINK1 function is also independent of its Hsp90 antagonizing activity. Instead, this effect is likely due to the combined abilities of celastrol to suppress PINK1 stabilization and perturb the association between PINK1 and TOM20. Our study reveals a novel biological activity of celastrol in mitochondrial regulation and a new cellular target of celastrol that can explain its mode of action.

PINK1 has been shown to form a 700 kDa complex with the TOM complex selectively on depolarized mitochondria (44), although the exact component(s) of the TOM complex that is involved in tethering PINK1 is not yet known. Tomm7 is required for accumulation of PINK1 at the OMM but is dispensable for protein import through the TOM complex into the matrix (13); however, no direct association between Tomm7 and PINK1 has been reported. Our results support the idea that TOM20 may be the primary binding partner of PINK1 and suggest a key role for TOM20 in PINK1 accumulation at the OMM. After CCCP treatment, PINK1 stays at the OMM upon loss of ∆ψm. Interestingly, celastrol treatment also results in a slow loss of ∆ψm, but it is insufficient to stimulate PINK1 accumulation at the OMM. Moreover, celastrol reverses the effects of CCCP on PINK1 accumulation at the OMM.

While celastrol suppresses the CCCP-induced rise of endogenous PINK1 protein levels in HeLa cells, it does not produce a similar reduction in PINK1 protein levels in MEF cells when hPINK1 is overexpressed. How hPINK1 functions is still disputed, as it fails to associate with TOM20 and forms high-molecular-weight aggregates. Based on these observations, a simple model that is consistent with our results is that celastrol prevents PINK1 localization and accumulation on the OMM, deterring subsequent Parkin activation (Figure 7G).
The action of celastrol further supports the importance of TOM20 and TOM70 in the stabilization of PINK1 on mitochondria when its import is disabled.

An alternative mechanism for suppression of mitochondrial damage-induced PINK1 elevation is through phosphorylation of MIC60 (mitofilin). Akabane et al. showed that PKA phosphorylation of MIC60, a component of a large protein complex critical for the formation of cristae junctions between inner and outer mitochondrial membranes, leads to a reduction in PINK1, although the exact mechanism has yet to be elucidated (49). Whether celastrol suppresses PINK1 elevation through the phosphorylation of MIC60 would be interesting to investigate in future studies. Finally, PINK1 is an unstably expressed protein, and the stabilization of elevated PINK1 protein levels requires sustained synthesis (50). Translation inhibition could deter PINK1 stabilization upon mitochondrial depolarization (50). Celastrol does not appear to affect PINK1 translation either in vitro or in vivo, and therefore is unlikely to be responsible for PINK1 suppression. Suppression of PINK1 elevation in HeLa cells could be a result of the rapid clearing of PINK1 aggregates.

Our study underscores the ability of celastrol to alter mitochondrial physiology, which is still poorly appreciated. The proposed model for celastrol’s mediation of the PINK1-Parkin mitophagy pathway via disruption of PINK1-TOM20 association adds to our understanding of the wide variety of reported physiological effects of celastrol. Recent studies have shown mitochondria to be important mediators in inflammatory responses (51, 52). Parkin has also been implicated in NF-κB activation and innate immunity (4, 53). The anti-inflammatory activity of celastrol has been long recognized, as has its activity in suppressing NF-κB activation (15,19). In traditional Chinese medicine, celastrol has been extensively used for treating arthritis. It will be interesting to investigate whether the anti-inflammatory effect of celastrol is functionally related to its effect on mitophagy. Another unexplained effect of celastrol is its ability to attenuate insulin resistance in type 2 diabetes (54). A more recent study observed that celastrol activates HSF1, which results in increased mitochondrial function in fat and muscle and protection against obesity, insulin resistance, and hepatic steatosis during high-fat diet regimens (55). Whether suppression of PINK1/Parkin-dependent mitophagy by celastrol is linked to the enhanced fat burning activity remains to be investigated.

Experimental Procedures

Cell Culture, stable cell lines and transfection
All cells were cultured using DMEM as previously described (11). HeLa cells were a gift from Sabrina Spencer. Lentiviral and retroviral production was performed in 293T cells using pHCMV-VSVg, pMDLg, pREV or pCL-Amp vectors as described (11). Stable cell lines infected with lentiviral or retroviral stocks were selected with 5 μg/mL blasticidin (Life Technologies), or 2 μg/mL puromycin (Sigma) for the given selection markers. Polyethylenimine (PEI) was used as the transfection reagent for viral packaging or transient transfections.

Constructs and RT-PCR
Venus-Parkin, RFP-Mito (previous known as RFP-Smac-MTS) and PINK1 expression vectors have been described (11). For the construction of C-terminal tagged PINK1, PINK1 was PCR-amplified and subcloned into pEGFP-N2 (Clontech) to obtain PINK1-EGFP. For recombinant protein expression, TOM20 (aa25-aa145) and TOM70 (aa111-aa608) were PCR amplified and cloned into pGEX-6P-1 and pET-15b respectively to derive pGEX-6P-GST-TOM20 and pET-15b-His6-TOM70. pRK5-PINK1 was made by subcloning of PINK1 into pRK5 between HindIII-Sall. Real-time PCR and quantification were performed as described previously (50). Briefly, RNA was isolated with TRIzol Reagent (Life Technologies). qPCR amplification was performed using Fast SybrGreen Master Mix (Applied Biosystems). The amplifications were performed in optical-grade 96-well plates on a StepOnePlus Real-Time PCR system. The CT was automatically determined by the instrument.

Recombinant protein expression and purification
pGEX-6P-GST-TOM20 and pET15b-His6-TOM70 were grown in E. coli BL21 (DE3), respectively. Bacteria were grown to OD 0.6 at 37°C. Then the temperature was decreased to 25°C and continued incubation in 200 μM IPTG for 12
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h. The bacteria pellets were collected and lysed before GST (GEhealth) or Ni-NTA (Qiagen) affinity chromatography following the manufacturer’s instructions. 35S-Met (PerkinElmer) labeled PINK1 was synthesized using the SP6 TNT Coupled Reticulocyte Lysate System (Promega) using pRK5-PINK1 as the template.

PINK1 kinase assay

PINK1 kinase assay using purified MBP-TcPINK1 was performed as described previously (34). In vitro kinase reactions were set up in a volume of 10 µL containing PINK1 kinase, 50 mM Tris-HCl (pH 8.0), 10 mM MgCl2, 160 µM cold ATP and 1 µCi [33P]-γ-ATP (PerkinElmer). The reactions were incubated at 30°C for 30 minutes. Reaction mixtures were resolved by SDS-PAGE and dried before PhosphorImaging (GEhealth).

Subcellular fractionation

Five million HeLa cells were harvested. The pellet was resuspended in 400 µl Tris Buffered Saline + 0.1% Tween 20 and rotated end over end for 60 min at 4°C. This mixture was homogenized using 7 passes of a 25 G needle and 1 ml syringe, then spun 20 min 4°C at 16,000g. The pellet was resuspended in 400 µl 0.5% Tween20 TBS on a rotator for 60 min 4°C. The mixture was spun at 16000g for 20 min 4°C. The supernatant was removed and stored as the Tween soluble fraction (insoluble non-mitochondria fraction). The pellet was resuspended in 400 µl 1% SDS TBS and boiled for 5 min 90°C, then sonicated with a probe sonicator. This pellet fraction was highly enriched with mitochondria.

Pull-down assay, immunoblotting, and antibodies

PINK1, TOM20 and TOM70 binding experiments were performed by incubation of 35S-PINK1 with purified GST-TOM20 and GST-TOM70 in the binding buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM Na3VO4, 25 mM β-glycerol-phosphate, 0.1 mM PMSF, and Complete Protease Inhibitor (Roche)) with 1 mL total volume at room temperature for 2 hr. Proteins collected on the GST or His beads were washed with binding buffer for three times prior to SDS-PAGE. Coomassie blue staining and autoradiography were used for detection of the proteins. The antibodies used in the immunoblotting are as follows: mouse anti-Parkin (1:1000 and 1:5000; clone PRK8, Sigma-Aldrich), rabbit anti-PINK1 (1:1000, D9G3, Cell Signaling), mouse anti-GAPDH(1:5000, Santa Cruz), mouse anti-PINK1 (1:1000, 8E10.1D6, Novus), rabbit anti-TOM20 (1:1000, 11802-1-AP, Proteintech).

Live cell imaging and fluorescence microscopy

High content imaging analysis was performed using ImageXpress XL (Molecular Devices). Cells were plated on 96-well Corning Costar 3603 imaging plates. Live cell imaging was collected for 0-8 hr as indicated. For high-resolution imaging, cells were grown on 4 well glass bottom chamber (Lab-Tek). Confocal images were acquired on a Nikon A1R Confocal using the 40X or 100X (NA 1.45) objective. To measure mitochondria membrane potential, cells were treated with 100 nM TMRE (tetramethylrhodamine, ethyl ester) dye (Life Technologies) at 37°C before the addition of tested chemicals. Imaging of TMRE staining of mitochondria in live cells was performed with a Nikon A1R confocal microscope or ImageXpress XL with excitation 549 nm and 575 nm emission filters.

Statistical analysis

Venus-Parkin mitochondrial recruitment was quantified by colocalization of Venus-Parkin with RFP-Smac-MTS in roughly 1000 cells per condition and from at least three independent experiments. Quantification was performed using MetaXpress application module Transfluor (Molecular Devices). For quantification of the mitochondrial cluster, more than 200 cells were quantified per condition. For quantification of PINK1 mitochondrial accumulation, images collected from Nikon A1R confocal microscope were used to obtain 100 cells with detectable PINK1-GFP signal on mitochondria. Standard deviations were calculated from at least three sets of data. The p values are determined using Microsoft Excel.

Small molecule compound library and chemicals

The small molecule compound libraries, including Approved Oncology Drugs Set and Structural Diversity Set were provided by NCI developmental therapeutics program (http://dtp.nci.nih.gov/branches/dscb/repo_open.html). The stock concentrations of the compounds are
10 mM and the final concentration of compounds used is indicated in the experiment. Celastrol was purchased from Cayman Chemical (Ann Arbor). Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) and cycloheximide were purchased from Sigma-Aldrich. Z-VAD-FMK was purchased from Abcam. 17-AAG was purchased from Cayman Chemicals. Gamitrinibtriphenylphosphonium (G-TPP) was a gift of Dr. Altieri (The Wistar Institute).

Native PAGE analysis

Precast 10% or 4-12% Norex™ Tris-Glycine mini gels 1.0 mm thick were purchased from ThermoFisher. For native PAGE analysis, samples were lysed in sample lysis buffer (20 mM Tris-HCL pH7.5, 150 mM NaCl, 10% glycerol, 1% NP40, 5 mM Na3VO4), incubated on ice for 20 min, and centrifuged at 12,000g for 5 min in cold room. Supernatants were mixed with 5x loading buffer (40% glycerol, 0.005% bromophenol blue, 2% sodium deoxycholate). Precast gels were pre-run at 200V for 70 min prior to loading the samples using a Mini Gel Tank (ThermoFisher, Cat. no. A25977). The buffer used for the cathode and anode are as follows: cathode (25 mM Tris, 192 mM Glycine, 0.4% deoxycholate), anode (25 mM Tris-HCl, 192 mM Glycine). Gels were typically run for 60 min at 200V and upon completion, gels were transferred using semi-dry before western blotting analysis.

Acknowledgments

We would like to thank Drs. Roy Parker, Sabrina Spencer and James Goodrich for critical readings of the manuscript and Drs. Natalie Ahn, Will Old, Amy Palmer, Ding Xue, and Hubert Yin for valuable discussions. We also thank Steve Wiltgen and Tim Baranowski of Molecular Devices for providing MetaXpress custom journals for quantification of Parkin mitochondrial translocation. We thank Dr. Altieri and Jae Ho Seo for sharing G-TPP. We thank Joseph Dragavon and the BioFrontiers Advanced Light Microscopy Core for their microscopy and imaging support. This work was supported GM113141 and in part by a grant from the National Institutes of Health R01CA107098 to X.L. E.B. was supported by a predoctoral training grant from NIGMS (T32GM08759). The ImageXpress MicroXL was supported by an NCRR grant S10 RR026680 and BDaria cell sorter was supported by S10OD021601 from NIH.

Conflict of Interest

The authors declare that they have no conflicts of interest with the content of this article.

Author Contributions

CZ, RW, ZL, EB, SL, MG and DC performed experiments and analyzed data. CZ, RW and XL designed studies. CZ, DC and XL wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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**Figure Legends**

**Figure 1.** Celastrol inhibits CCCP-induced mitochondrial translocation of Parkin. (A) HeLa cells expressing Venus-Parkin-WT and RFP-Mito, a mitochondrial targeting signal from Smac fused with RFP, were treated with CCCP (20 µM) in the presence or absence of Celastrol (10 µM). Scale bar, 10 µm. (B) These data were used to calculate the IC_{50} of celastrol inhibition of CCCP-induced Parkin mitochondrial recruitment.

**Figure 2.** Pharmacological disruption of the microtubule network does not affect CCCP-induced Parkin mitochondrial translocation in the presence or absence of celastrol. Time-lapse imaging of HeLa-PM cells using an A1R confocal microscope every 10 min. The montages are: (A) Vehicle only. (B) 5 µM nocodazole. (C) 10 µM Celastrol. (D) 10 µM CCCP. (E) 10 µM CCCP plus 10 µM Celastrol. (F) 10 µM CCCP plus 5 µM nocodazole. Scale bar, 10 µm.

**Figure 3.** Celastrol inhibits CCCP-induced activation of endogenous PINK1 levels and Parkin phosphorylation in HeLa cells. (A-B) Immunoblotting of the endogenous PINK1 and ectopically expressed Parkin in HeLa cells treated with CCCP (20 µM), Celastrol (10 µM), or both and analyzed over time (A), or across increasing dose of celastrol (B). (C) Steady-state levels of PINK1 were analyzed in the presence or absence of celastrol and/or cycloheximide to assess the metabolic stability of PINK1. (D) Similar experiments as in (C) were conducted in HeLa cells exposed to CCCP in the presence or absence of either celastrol or 17-AAG. (E) Venus-Parkin subcellular localization was visualized by live cell microscopy in HeLa Venus-Parkin-WT cells exposed to either celastrol or 17-AAG in the presence or absence of CCCP. Scale bar, 10 µm. (F) Quantification of (E) (n=3; *p<0.0001, student's unpaired t-test). (G) RFP-Mito and PINK1-EGFP expressing HeLa cells exposed to CCCP and/or Celastrol were assessed for subcellular fluorophore colocalization via live cell microscopy. Scale bar, 10 µm. (H) Mean GFP intensity of HeLa cells expressing RFP-Mito and PINK1-EGFP was normalized to mean RFP intensity and calculated as a function of time in cells exposed to CCCP and/or celastrol. (I) TMRE stained HeLa cells exposed to CCCP and/or Celastrol were assessed for mean fluorescence as a function of time.

**Figure 4.** Celastrol inhibits PINK1-dependent Parkin mitochondrial translocation induced by G-TPP. RFP-Mito and Venus-Parkin subcellular distribution was visualized (A) and quantified (B) in HeLa cells exposed to Celastrol (5 µM) and/or G-TPP (10 µM) via live cell confocal microscopy. Scale bar, 10 µm. (C) Endogenous PINK1 and ectopically expressed Parkin, Ser65 phospho-ubiquitin levels were measured using immunoblotting of HeLa whole cell lysates from cells exposed to celastrol and/or G-TPP for the indicated times.

**Figure 5.** Celastrol inhibits CCCP induced mitophagy. RFP-Mito and Venus-Parkin subcellular distribution were visualized by live cell microscopy for representative cells at the indicated times (A) and quantified (B) in HeLa exposed to CCCP and/or celastrol. Scale bar, 10 µm. (C) Immunoblotting of TOM20 in HeLa-PM cells treated with CCCP and/or celastrol for 12 hr. (D) HeLa cells expressing CFP-LC3, Venus-Parkin and RFP-Mito were used in live cell microscopy experiments to assess the colocalization of Venus/RFP and CFP/RFP subcellular distribution as a function of time and treatment with CCCP and/or celastrol. (E) Immunoblotting of CFP-LC3 in HeLa cells exposed to identical conditions as (D).

**Figure 6.** Celastrol inhibits CCCP-induced Parkin mitochondrial translocation and function in MEF cells overexpressing exogenous human PINK1 protein. (A) Immunoblotting analysis of PINK1 levels in PINK1-MEF cells treated with CCCP (20 µM) and/or celastrol (2.5 µM) for indicated time course (0, 2,
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4, 6 h). (B) Immunoblotting analysis of PINK1 in whole cell lysates of PINK1-MEF cells treated with CCCP (20 µM) in the presence or absence of celastrol (2.5 µM) for indicated time course (0, 1, 2 h). (C) Confocal microscopy visualization of the subcellular distribution of Venus-Parkin in MEF cells expressing human PINK1 and human Venus-Parkin and treated with CCCP and/or celastrol at the indicated times. Scale bar, 10 µm. Quantification of images in (C) is displayed in (D). (E) Immunoblotting analysis of PINK1-Flag levels in PINK1-Flag-MEF cells treated with CCCP and/or celastrol for 2 hours. (F) Immunoblotting analysis of fractionated insoluble and soluble lysate fractions following treatment of cells with CCCP and/or celastrol for 2 hours and subsequent lysing and centrifugation fractionation using phase separation (50,000 RCF). (G) Native and SDS-PAGE analysis of the PINK1 containing complexes in cells treated with CCCP and/or celastrol for 2 hours.

Figure 7. Celastrol disrupts the interaction between PINK1 and TOM20 in vitro and in vivo. (A) Analysis of in vitro PINK1 interaction with GST-Tom20 and GST-TOM70 using a GST pull-down assay with 35S-Met labeled PINK1 in the presence or absence of celastrol (10 µM). Densitometry quantification of the radiograph (upper panel) in (A) is displayed in (B). (C) A similar GST-Pulldown assay was used to assess the relative interaction between 35S-Met labeled PINK1 and GST-TOM20 as a function of increasing celastrol concentration. (D) Autophosphorylation of recombinant MBP-TcPINK1 as a function of increasing celastrol dose was measured in an in vitro kinase assay using 33P-γ-ATP. (E) Analysis of celastrol’s (10 µM) ability to disrupt the in vitro interaction between GST-Tom20 and His-Tom70 using a GST pull-down assay. (F) Analysis of the dependence of the in vitro interaction between GST-TOM20 and 35S-Met labeled PINK1 on RNA using a GST pull-down assay in the presence or absence of RNase A. (G) Analysis of co-immunoprecipitation of exogenous PINK1-Flag with endogenous TOM20 in whole cell lysates from MEF-PINK1-Flag cells exposed to CCCP and/or celastrol for 2 hours. (H) A schematic model for celastrol inhibition of the PINK1-Parkin pathway.
**Figure 1**

**A**

Images showing the effect of different treatments (DMSO, CCCP, CCCP + Celastrol) on Venus-Parkin and RFP-Mito at 0 min and 90 min. The merge column shows the combined images.

**B**

Graph depicting the percentage of Parkin on mitochondria (% Parkin on mitochondria) against Celastrol concentration (μM). The IC$_{50}$ is indicated as 2.5 ± 0.07 μM.
The plant triterpenoid celastrol blocks PINK1-dependent mitophagy by disrupting PINK1's association with the mitochondrial protein TOM20

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J. Biol. Chem. published online March 18, 2019

Access the most updated version of this article at doi: 10.1074/jbc.RA118.006506

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