Constitutive Activation of PINK1 Protein Leads to Proteasome-mediated and Non-apoptotic Cell Death Independently of Mitochondrial Autophagy

Received for publication, January 8, 2016, and in revised form, May 19, 2016. Published, JBC Papers in Press, June 14, 2016, DOI 10.1074/jbc.M116.714923

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Phosphatase and tensin homolog-induced putative kinase 1 (PINK1), a Ser/Thr kinase, and PARKIN, a ubiquitin ligase, are causal genes for autosomal recessive early-onset parkinsonism. Multiple lines of evidence indicate that PINK1 and PARKIN cooperatively control the quality of the mitochondrial population via selective degradation of damaged mitochondria by autophagy. Here, we report that PINK1 and PARKIN induce cell death with a 12-h delay after mitochondrial depolarization, which differs from the time profile of selective autophagy of mitochondria. This type of cell death exhibited definite morphologic features such as plasma membrane rupture, insensitivity to a pan-caspase inhibitor, and did not involve mitochondrial permeability transition. Expression of a constitutively active form of PINK1 caused cell death in the presence of a pan-caspase inhibitor, irrespective of the mitochondrial membrane potential. PINK1-mediated cell death depended on the activities of PARKIN and proteasomes, but it was not affected by disruption of the genes required for autophagy. Furthermore, fluorescence and electron microscopic analyses revealed that mitochondria were still retained in the dead cells, indicating that PINK1-mediated cell death is not caused by mitochondrial loss. Our findings suggest that PINK1 and PARKIN play critical roles in selective cell death in which damaged mitochondria are retained, independent of mitochondrial autophagy.

Phosphatase and tensin homolog-induced putative kinase 1 (PINK1) and PARKIN are causal genes for autosomal recessive early-onset parkinsonism (1). PINK1 is a unique Ser/Thr kinase localized on the outer membrane of damaged mitochondria, where it is subsequently autophosphorylated, followed by the formation of a larger protein complex that contains a translocon of the outer membrane (TOM) complex (2–4). PINK1 localization on damaged mitochondria selectively recruits PAR- KIN (5, 6), and phosphorylates PARKIN to uncover latent ligase activity (7). PINK1 and phosphorylated PARKIN share a cooperative role to modify mitochondrial outer membrane proteins with phospho-ubiquitin chains, and mitochondria decorated by poly-ubiquitin chains are eliminated by selective mitochondrial autophagy (1), thereby maintaining mitochondrial quality.

Programmed cell death serves fundamental functions in tissue development and homeostasis and is associated with several human pathologies, including neurodegeneration, autoimmune diseases, and cancer (8). Apoptosis, the best studied form of programmed cell death, is characterized by cell shrinkage, blebbing, nuclear fragmentation, and chromatin condensation, and it requires caspase activation (9). Many studies have revealed caspase-independent but genetically regulated forms of cell death that are classified according to their distinct morphologic features and specific inhibitors (10). PARKIN prevents cells from dying in response to proapoptotic stimuli (11, 12). The cytoprotective effects of PARKIN are relatively accepted because loss of PARKIN function leads to progressive degeneration of dopaminergic neurons, giving rise to Parkinson disease. The roles of PINK1 and PARKIN in programmed cell death caused by non-apoptotic triggers, however, remain poorly characterized.

Recently, we reported that removal of a conventional mitochondrial targeting sequence corresponding to the N-terminal 34-amino acid residues allows PINK1 to translocate to the outer membrane in an unconventional signal-dependent manner and induces the autophosphorylation of PINK1 and translocation of PARKIN without mitochondrial depolarization (13). The truncated PINK1 is constitutively active, can recruit PARKIN to the mitochondria, and promotes subsequent events, even when the mitochondria are energized. Here, we utilized both a chemical uncoupler to depolarize mitochondria and a constitutively active form of PINK1 to reveal that PINK1 activation caused cell death that did not involve caspase activation or mitochondrial permeability transition (MPT), and we induced definite morphologic features, such as plasma membrane rupture. Cell death was induced with a 12-h delay after mitochondrial permeability transition; CCCP, carbonyl cyanide m-chlorophenylhydrazone; ROS, reactive oxygen species; PARP, poly(ADP-ribose) polymerase; KD, kinase-dead; CA, constitutively active; CFP, cyan fluorescent protein.
depriving mitochondria of membrane potential, which differs from the time profile of selective autophagy of mitochondria. Importantly, autophagic activity was dispensable for the cell death induced by PINK1 activation, and mitochondria were still retained in the dead cells. Proteasomal activity, however, was crucial for the PINK1-induced cell death. Our findings suggest that PINK1 and PARKIN regulate not only mitochondrial clearance but also proteasome-dependent cell death with different durations of mitochondrial depolarization.

Results

PARKIN-dependent Cell Death in Response to Mitochondrial Depolarization—In normal culture conditions with a high glucose concentration, HeLa cell morphology is not significantly altered by treatment with the protonophore CCCP, because cancer cells mainly utilize glycolysis to produce ATP (14). In contrast to HeLa cells lacking endogenous CCCP, because cancer cells mainly utilize glycolysis to produce ATP (14). In contrast to HeLa cells lacking endogenous PARKIN expression (Fig. 1C, left panel) (15), HeLa cell lines expressing exogenous PARKIN were sensitive to CCCP treatment. To examine whether PARKIN expression influences cell survival in the presence of CCCP, two independent cell lines stably expressing exogenous PARKIN were cultured for 48 h as described in A, and the ratio of dead cells in the populations was calculated by measuring the activities of proteases released from cells that lost their membrane integrity. E. control HeLa and HA-PARKIN-expressing HeLa cells were cultured for the indicated times in the presence of CCCP and stained with PI. F. HA-PARKIN-expressing HeLa cells cultured for 48 h with either DMSO (panels a and b) or 10 μM CCCP (panels c–f), and then stained with PI. Live images were obtained using fluorescence microscopy with a fluorescence optical sectioning system. Scale bar, 10 mm. C. total cell lysates prepared from SH-SY5Y, HEK293, control HeLa, and PARKIN-expressing HeLa cell lines were analyzed by immunoblotting with antibodies to PARKIN and actin as a loading control. Asterisk indicates endogenous PARKIN. D. control HeLa and HeLa cell lines expressing exogenous PARKIN were cultured for 40 h as described in A, and the ratio of dead cells in the populations was calculated by measuring the activities of proteases released from cells that lost their membrane integrity. E. control HeLa and HA-PARKIN-expressing HeLa cells were cultured for the indicated times in the presence of CCCP and stained with PI. F. HA-PARKIN-expressing HeLa cells cultured for 48 h with either DMSO (panels a and b) or 10 μM CCCP (panels c–f) were fixed, and thin sections were visualized by electron microscopy. Most of the CCCP-treated cells had disrupted plasma membranes (panels c and d). Membrane integrity was maintained in a small population of the cells (panels e and f). Lower panels b, d, and f show high magnification images of the corresponding cell in the upper panels. Arrow indicates a mitochondrion. Scale bars, 10 μm (panels a, c, and e) and 1 μm (panels b, d, and f). G, SH-SY5Y and HEK293 cells were cultured as described in A and then stained with PI. H, SH-SY5Y and HEK293 cells were cultured for the indicated times in the presence of CCCP and stained with PI. Data in A, D, E, G, and H represent the mean ± S.E. of three independent experiments (n=100 individual cells were counted). *, p<0.05; **, p<0.005; *** p<0.0005 (Student’s t test).
Mitochondrial Autophagy Is Induced Prior to PARKIN-Dependent Cell Death—Mitochondrial depolarization leads to autophagy of damaged mitochondria through PARKIN activation (16). To investigate how the cell viability reduction relates to autophagy, the time profile of the mitochondrial autophagic activity was analyzed using a mitochondria-targeted and pH-sensitive fluorescent protein (mito-Keima) (17). When mitochondria were depolarized in HA-PARKIN-expressing cells, cytochrome c, a mitochondrial protein, was delivered to the acidic compartments, which were marked by the lysosomal

PARKIN revealed that the CCCP-treated cells were stained with PI (Fig. 1B). The number of stained HA-PARKIN cells increased with a 12-h delay after the addition of CCCP (Fig. 1E). Thus, exposure to CCCP reduced the viability of PARKIN-expressing HeLa cells. Similar results were obtained in a luminescence cytotoxicity assay that measures the activities of proteases released from damaged cells (Fig. 1D).

Electron microscopic analysis revealed a loss of plasma membrane integrity in most HA-PARKIN-expressing cells cultured in the presence of CCCP (Fig. 1F, panel c). Compared with DMSO-treated cells, the morphology of HA-PARKIN-expressing cells was not significantly altered, i.e. no cell shrinkage or swelling was observed. Importantly, the nucleus appeared to be intact and not fragmented. Although plasma membrane integrity was maintained in some of the cells after CCCP treatment, nuclear fragmentation and chromatin condensation were not detected in such cells (Fig. 1F, panel e). The number of mitochondria was clearly decreased by CCCP treatment, but mitochondria were observed even in cells in which the plasma membrane integrity was lost (Fig. 1F, panel d, arrow). In SH-SYSY and HEK293 cells, which express endogenous PARKIN, CCCP treatment caused cell death, as expected (Fig. 1G). The number of PI-stained cells increased with a significant delay after the addition of CCCP, like in the PARKIN-expressing HeLa cell lines (Fig. 1H). The significantly lower number of PI-stained HEK293 cells compared with SH-SY5Y cells may be due to differences in the expression of endogenous PARKIN (Fig. 1C, left panel). These findings indicated that the expression of PARKIN induced cell death with an obvious delay in response to mitochondrial depolarization.

**FIGURE 2.** Mitochondrial autophagy is activated prior to PARKIN-induced cell death. A, HeLa cells stably expressing both HA-PARKIN and mito-Keima were transfected with LAMP1-EGFP and incubated for 4 h with either DMSO (panels a–f) or 10 μM CCCP (panels g–l). Live images were obtained using fluorescence microscopy. Fluorescent signals of mito-Keima existing in an acidic environment were observed by excitation using 560-nm light. Lower panels d–f and j–l show high magnification images of the corresponding cell in the upper panels. Arrows indicate mito-Keima in LAMP1-positive compartments. Scale bars, 10 μm. B, HA-PARKIN-expressing cells were transfected with LAMP1-EGFP, incubated for 8 h with either DMSO (panels a–f) or 10 μM CCCP (panels g–l) in the presence of 20 μM chloroquine, fixed with paraformaldehyde, and subjected to immunofluorescence analysis using anti-cytochrome c antibody. Lower panels d–f and j–l show high magnification images of the corresponding cell in the upper panels. Arrows indicate cytochrome c in the LAMP1-positive compartments. Scale bars, 10 μm. C, HA-PARKIN-expressing HeLa cells were transfected with mito-Keima and cultured for the indicated times in the presence of 10 μM CCCP. Live images were obtained using fluorescence microscopy. Fluorescent signals of mito-Keima in neutral and acidic pH environments were detected by excitation using 430 and 560 nm of light, respectively. D, HA-PARKIN-expressing cells were transfected with mito-Keima and incubated with either DMSO or 10 μM CCCP for the indicated times. Cells with more than 15 fluorescent dots of mito-Keima in acidic compartments were scored as an index of mitochondrial autophagy. E and F, HA-PARKIN-expressing cells were incubated with 10 μM CCCP for the indicated times and analyzed by immunofluorescence microscopy (E) and immunoblot (F) using antibodies to an outer membrane protein TOM20, a matrix protein mHSP70, and a loading control protein p97. Intensity in F represents normalized values of the corresponding band intensity. Data in D and E represent the mean ± S.E. of three independent experiments (>100 individual cells were counted).
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protein LAMP1 (Fig. 2B). Similarly, intracellular fluorescent dots of mito-Keima excited by 560 nm of light were observed in the LAMP1-positive compartments upon treatment with CCCP (Fig. 2A), as reported previously (17, 18). Therefore, fluorescent dots of mito-Keima excited by 560 nm of light were scored as mitophagic activity. The number of cells with mitophagy dots increased in proportion to the duration of CCCP incubation and reached a plateau after a 12-h incubation (Fig. 2, C and D). Consistently, the number of cells with the mitochondrial proteins TOM20 and mtHSP70 was decreased by a 12-h incubation with CCCP and did not change afterward (Fig. 2E). Similar results were obtained by immunoblot analysis (Fig. 2F). In the plateau phase, mitophagic activity appeared not to increase, because the protein levels of the mitochondrial proteins were not significantly altered after a 12-h incubation with CCCP. These results suggested that PARKIN-mediated mitochondrial autophagy was increased during the initial 12-h incubation. Taken together with the time profile of PI staining (Fig. 1E), the decrease in cell viability began after mitochondrial autophagy in response to mitochondrial depolarization. Thus, PARKIN-dependent cell death was induced with a significant delay, which differed from the time profile of mitochondrial autophagy.

PARKIN-mediated Cell Death Does Not Depend on Caspase Activity and MPT—To determine whether CCCP-induced death of PARKIN-expressing cells is an apoptotic event, a pan-caspase inhibitor, Z-VAD-fmk, was added with the CCCP. The number of dead cells, however, was unaffected by 100 μM Z-VAD-fmk (Fig. 1, A and D). At this concentration, Z-VAD-fmk completely blocks actinomycin D-induced apoptosis in HeLa cells (19). Addition of a pan-caspase inhibitor to HEK293 and SH-SY5Y cells also failed to prevent CCCP-induced cell death (Fig. 1G). To further explore the steps of the apoptotic process, we examined the release of cytochrome c from mitochondria. Immunofluorescence microscopic analysis revealed a marked release of cytochrome c in both control and PARKIN-expressing HeLa cells upon treatment with actinomycin D, although treatment with CCCP barely induced cytochrome c release (Fig. 3, A and B), which was consistent with the delivery of cytochrome c to the acidic compartments upon mitochondrial depolarization (Fig. 2B). Moreover, immunoblot analysis indicated that activation of caspase-3, an effector caspase, and processing of poly(ADP-ribose) polymerase (PARP), a well known substrate cleaved by caspase, were undetectable upon treatment with CCCP (Fig. 3C), consistent with the previous finding of unprocessed caspase-3 in primary cortical neurons upon CCCP treatment (20). The results clearly demonstrated that caspase activity was not required for PARKIN-mediated cell death in response to mitochondrial depolarization. Mcl-1 is a mitochondrial protein belonging to the Bcl-2 family. Its rapid degradation by the ubiquitin-proteasome system is crucial for initiating the apoptotic pathway (21). As expected, actinomycin D induced complete degradation of Mcl-1. Although Mcl-1 was partially reduced by CCCP treatment, the CCCP-induced reduction of Mcl-1 did not differ significantly between control and PARKIN-expressing HeLa cells (Fig. 3C), suggesting that CCCP-stimulated degradation of Mcl-1 was not sufficient to induce PARKIN-mediated cell death.

Bcl-xL, a Bcl-2 family member, is a well characterized antiapoptotic protein (22). In the HeLa cell line stably expressing Bcl-xL, activation of the apoptotic pathway via mitochondria was suppressed (Fig. 3, D and E). Even in the Bcl-xL-expressing cell line, GFP-PARKIN expression induced cell death when cells were incubated with CCCP (Fig. 3F). Thus, Bcl-xL expression completely blocked apoptosis but did not block PARKIN-mediated cell death. Taken together with the dispensability of caspase activities, the results suggest that the PARKIN-mediated cell death was a non-apoptotic event. It should be noted that the effect of GFP-PARKIN expression in cells expressing Bcl-xL was moderate compared with that in control HeLa cells. This moderate effect might be due to a slight reduction in mitochondrial targeting of GFP-PARKIN in Bcl-xL-expressing cells (Fig. 3G), consistent with a previous report that Bcl-2 family members attenuate the translocation of PARKIN to the mitochondria (23). Nonetheless, PARKIN-mediated cell death was not completely blocked by Bcl-xL at an expression level sufficient to inhibit apoptotic events.

Treatment with CCCP in human cell lines induces ROS production (24). Oxidative stresses, including ROS generation, appear to be related to MPT (25), which leads to necrosis and apoptosis (26, 27). To examine whether PARKIN-mediated cell death involves MPT, ROS production was first measured in HA-PARKIN-expressing cells. ROS production transiently increased after exposure to CCCP (Fig. 3H). Next, a calcium ionophore, A23187, was used to examine the involvement of MPT because overload of cytosolic calcium causes MPT (26). Prolonged exposure of A23187 induces apoptotic cell death in HeLa cells (28). PARKIN-expressing HeLa cells were incubated with A23187 for 30 h and stained with PI, because A23187 induces apoptosis of HeLa cells after a 48-h incubation (28). Upon treatment with A23187, the number of PI-stained cells did not increase even in HA-PARKIN-expressing cells (Fig. 3I). Moreover, the MPT inhibitors, cyclosporine A and bongrekic acid, hardly blocked CCCP-induced cell death in HA-PARKIN-expressing cells (Fig. 3J). These results indicated that MPT was not required for PARKIN-mediated cell death, although ROS generation transiently occurred in response to mitochondrial depolarization. ATP depletion facilitates MPT through alterations in calcium homeostasis (29). Intracellular ATP levels were measured when PARKIN-expressing cells were treated with CCCP. Intracellular ATP was not altered until 24 h after the addition of CCCP and later decreased (Fig. 3K). This result indicated that ATP deprivation occurred after cell death and supported the notion that MPT was not necessary for PARKIN-mediated cell death.

Constitutively Active Form of PINK1 Induces Cell Death in an Autophosphorylation-dependent Manner—PARKIN activation depends on PINK1 (5, 6). To examine the requirement of PINK1 for PARKIN-mediated cell death, PINK1 was knocked down in control and PARKIN-expressing HeLa cells. More than 90% knockdown of PINK1 was confirmed by immunoblot analysis (Fig. 4A). Unexpectedly, PINK1 knockdown induced caspase-dependent cell death in control HeLa cells upon prolonged exposure to CCCP (Fig. 4B, left panel). Introduction of another PINK1-targeted siRNA also caused CCCP-induced cell death, indicating that PINK1 knockdown alone was responsible for CCCP-induced and caspase-dependent cell death in control
HeLa cells. Caspase insensitivity of PARKIN-mediated cell death was confirmed even when transfected with control siRNA. In HA-PARKIN-expressing cells, PINK1 knockdown significantly suppressed CCCP-induced cell death in the presence of Z-VAD-fmk, whereas PINK1 silencing had no effect on the cell death without the pan-caspase inhibitor (Fig. 4B, right panel). The results indicated that PARKIN-mediated and caspase-insensitive cell death required PINK1, although PINK1 down-regulation induced another type of cell death that was not mediated by PARKIN.

PINK1(ΔN34), a truncated form that lacks the mitochondria-targeting sequence, is the constitutively active form of PINK1, because removal of the typical mitochondria-targeting sequence at the N terminus makes PINK1 constitutively active (13). To examine whether introducing PINK1(ΔN34) induces cell death that is not affected by caspase inactivation, we transfected HA-PARKIN-expressing cells with PINK1(ΔN34) or PINK1(ΔN34, KD), which carries the kinase-dead (KD) mutation (K219A, D362A, and D364A) to abolish PINK1 kinase activity, together with GFP as a marker.

FIGURE 3. PARKIN-mediated cell death does not depend on caspase activity and mitochondrial permeability transition. A, control HeLa cells and HA-PARKIN-expressing cells were cultured for 8 h with either DMSO, 10 μM CCCP, or 20 μM actinomycin D (ActD) in the presence of 100 μM Z-VAD-fmk, and the release of cytochrome c from mitochondria was analyzed by immunofluorescence microscopy. B, HA-PARKIN-expressing cells were cultured as described in A, fixed, and immunostained with antibodies to cytochrome c and mitofilin. The white arrowheads indicate cells with cytochrome c released from mitochondria. Scale bar, 10 μm. C, control HeLa cells and HA-PARKIN-expressing cells were transfected for 12 h for with DMSO, CCCP, or ActD and subjected to immunoblotting with antibodies to PARP, cleaved PARP, caspase-3, cleaved caspase-3, Mcl-1, or p97 as a loading control. D, control HeLa and Bcl-xL-expressing HeLa cells were cultured for 8 h with either DMSO or 20 μM ActD in the presence of 100 μM Z-VAD-fmk, and the release of cytochrome c from mitochondria was analyzed by immunofluorescence microscopy. E, Bcl-xL-expressing cells were transfected with plasmids carrying GFP or GFP-PARKIN, incubated with 10 μM CCCP for 0, 24, or 48 h, and stained with PI. F, HA-PARKIN-expressing cells were transfected with plasmid carrying GFP-PARKIN and treated for 60 min with DMSO or 10 μM CCCP. Recruitment of GFP-PARKIN to the mitochondria was analyzed by immunofluorescence microscopy. H, HA-PARKIN-expressing cells were cultured for indicated times in the presence of either DMSO or CCCP. ROS production was measured and normalized by total amounts of protein. I, control HeLa cells and Bcl-xL-expressing cells were transfected with plasmids carrying GFP or GFP-PARKIN, and intracellular ATP was measured and normalized by total amounts of protein. Data in A and D–K represent the mean ± S.E. of three independent experiments (N=100 individual cells were counted). *, p<0.05; **, p<0.005 (Student’s t test). n.s. represents not significant.
sion induced Z-VAD-fmk-insensitive cell death without the mitochondrial uncoupler, whereas PINK1(H9004N34, KD) expression failed to induce cell death (Fig. 4, C and D). Furthermore, ectopic expression of PINK1(H9004N34) did not affect mitochondrial membrane potential (Fig. 4I), indicating that PINK1(H9004N34) induced caspase-independent cell death irrespective of the mitochondrial membrane potential.

Dual phosphorylation at two serine residues (Ser-228 and Ser-402) of PINK1 is crucial for PARKIN recruitment (2). To assess the effect of PINK1 autophosphorylation on cell death, we evaluated the effects of PINK1(H9004N34) mutants with an alanine substitution at one or both phosphorylation sites (Fig. 4E). Deficient autophosphorylation of the PINK1(H9004N34) mutants was confirmed by phosphate-affinity SDS-PAGE (Phos-tag SDS-PAGE) and immunoblot analysis (Fig. 4F). As expected, mutants with the single alanine substitution at either Ser-228 or Ser-402 were partially phosphorylated, whereas mutants with double substitutions (S228A and S402A) had no detectable phos-
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Phosphorylation. Expression of PINK1(ΔN34) caused cell death with a significant delay (Fig. 4G). In contrast, the autophosphorylation-deficient mutant PINK1(ΔN34, S228A, S402A), as well as PINK1(ΔN34, KD), failed to induce cell death. Interestingly, expression of partially phosphorylated mutants (PINK1(ΔN34, S228A) and PINK1(ΔN34, S402A)) induced only a moderate level of cell death, suggesting that the level of PINK1 activity correlated with cell death. Next, to assess the inhibitory effects of the autophosphorylation-deficient and KD mutants on cell death, the numbers of PI-stained cells expressing the PINK1(ΔN34) mutants were examined with or without CCCP treatment. CCCP-induced cell death was not inhibited by PINK1(ΔN34, KD) or PINK1(ΔN34, S228A, S402A) mutants (Fig. 4H), indicating that neither autophosphorylation-deficient nor kinase-dead PINK1 mutants had a dominant negative effect on PARKIN-mediated cell death. The results indicated that the constitutively active form of PINK1 (PINK1-CA) resulted in cell death even without mitochondrial depolarization.

PINK1-CA-induced Cell Death Requires PARKIN Ubiquitin Ligase Activity and TOM20—PINK1 stimulates both enzymatic activity and translocation of PARKIN (5, 6). To address whether PARKIN ubiquitin ligase activity is directly necessary for the PINK1-CA-induced cell death, we evaluated two PARKIN mutants (T415N and G430D) that are able to translocate to the mitochondria but have no enzymatic activity (6). The GFP-PARKIN mutants and PINK1-CA were co-transfected into HeLa cells to assess cell death. Both of the PARKIN mutants barely induced cell death (Fig. 5A), indicating that ubiquitination by PARKIN was required for cell death.

PINK1 forms a large protein complex containing the TOM complex (3, 4), which acts as a receptor and translocation pore for mitochondrial preproteins. Furthermore, the recruitment of PINK1-CA to the mitochondria requires the TOM complex (13). To verify whether the TOM complex contributes to cell death induced by PINK1-CA, we knocked down TOM20, a subunit of the TOM complex, and confirmed its down-regulation by immunoblot analysis (Fig. 5C). TOM20 knockdown drastically reduced PINK1-CA-mediated cell death (Fig. 5B). Immunofluorescence microscopy indicated that mitochondrial targeting of PINK1-CA was abolished by TOM20 knockdown (Fig. 5D). Thus, the TOM complex contributed to cell death by recruiting PINK1-CA to the mitochondria.

Programmed necrosis, termed necroptosis, is induced by combined treatment with TNF-α, a Smac mimic, and Z-VAD-fmk (30). Necroptosis involves rupture of the plasma membrane but not nuclear fragmentation, similar to the morphologic features of PINK1-mediated cell death. Therefore, we addressed whether cell death induced by PINK1-CA was related to necroptosis. The addition of inhibitors of programmed necrosis, necrosulfonamide and necrostatin-1 (30–32), did not block cell death induced by PINK1-CA (Fig. 5E). Moreover, knockdown of MLKL, a key component of programmed necrosis (32), did not affect cell death induced by PINK1-CA (Fig. 5, F and G). Thus, PINK1-CA-induced cell death was independent of MLKL-mediated programmed necrosis.

Proteasomal Activity, but Not Autophagy, Is Crucial for PINK1-C-induced Cell Death—Activation of PARKIN on mitochondria allows the outer membrane proteins to be ubiquitinated and promotes the subsequent steps: degradation of outer membrane proteins by proteasomes and clearance of damaged mitochondria by autophagy (16, 33). To determine whether proteasomes contribute to cell death induced by PINK1-CA, we applied two proteasome-specific inhibitors, lactacystin and bortezomib. Addition of the inhibitors significantly reduced the number of PI-stained cells with PINK1(ΔN34) (Fig. 6A), indicating that impairment of proteasomal activity blocked the PINK1-CA-induced cell death. Proteasomes function in diverse cellular processes (34); thus their activity often influences cell survival. Under these conditions, PI staining of PINK1(ΔN34, KD)-transfected cells was unaffected by the proteasome inhibitors, suggesting that treatment with these inhibitors did not induce other types of cell death. Similarly, PINK1-CA–mediated cell death in HEK293 cells was also suppressed by treatment with lactacystin (Fig. 6B). By vital staining using another membrane-impermeable dye, Zombie Green, proteasome inhibitor-dependent suppression of PINK1-CA–induced cell death was confirmed in PARKIN-expressing HeLa cells (Fig. 6C). This fluorescent dye is amine-reactive and has a different property from PI, which preferentially binds to DNA. The similar results obtained using two different dyes suggested that proteasome inhibitors suppressed the PINK1-CA–induced cell death rather than the membrane permeability of fluorescent dyes. Moreover, total proteasome activities were elevated when PINK1(ΔN34) was introduced (Fig. 6D), indicating that expression of PINK1-CA induced proteasome activation. Taken together, the results indicated that PINK1-CA–mediated cell death depended on proteasomal activity.

Autophagic activity transiently increased prior to the PARKIN-mediated cell death (Fig. 2, C and D), suggesting that the cell death was a distinct process from autophagy. To confirm that autophagy was not involved in the cell death, we utilized autophagy-deficient HeLa cell lines (FIP200 KO and ATG7 KO). Two independent clones of each knocked out cell line were examined by co-transfection of GFP-PARKIN with either PINK1(ΔN34) or PINK1(ΔN34, KD). None of the tested autophagy-deficient cell lines exhibited significant mitophagic activity under the hypoxia condition (Fig. 6E), in which mitochondria-selective autophagy is induced (17). Furthermore, the autophagy-deficient cell lines virtually lost mitophagic activity induced by the introduction of PINK1(ΔN34) and GFP-PARKIN (Fig. 6F). The number of PI-stained cells deficient in autophagy was not significantly reduced compared with that of control HeLa cells (Fig. 6G). In particular, the number of PI-stained cells with FIP200 KO was similar to that of control HeLa cells. Thus, autophagy was dispensable for PINK1-CA–mediated cell death. It should be noted that DNA transfection appeared to be slightly cytotoxic only to the ATG7 KO cell line. Gene disruption of ATG7, however, did not suppress the cell death induced by PINK1-CA. The dispensability of autophagy raised the possibility that mitochondrial clearance is not necessary for PINK1-CA–mediated cell death. Indeed, the PINK1(ΔN34)-transfected cells, in addition to PI staining, exhibited mitochondrial staining by MitoTracker Green, a fluorescent dye that accumulates in the mitochondria irrespective of their membrane potential (Fig. 6H, panel e). Similarly, mitochondrial staining by MitoTracker Green was detectable in
CCCP-treated cells with HA-PARKIN (Fig. 6H, panel b), which was supported by electron microscopic analysis (Fig. 1F, panels c and d). Taken together with the requirement for proteasomal activity, these findings suggest that PINK1-CA-induced cell death requires the degradation of mitochondrial outer membrane proteins by proteasomes but not the elimination of mitochondria by autophagy.

**Discussion**

Activation of PINK1 triggers the selective removal of damaged mitochondria in a PARKIN-dependent manner (1). This study demonstrated that treatment with an uncoupler to depolarize the mitochondria enhanced the PINK1- and PARKIN-dependent cell death with definite morphologic features. Furthermore, prolonged expression of PINK1-CA also caused cell death irrespective of the mitochondrial membrane potential. Although mitochondrial depolarization leads to the impairment of a variety of cellular processes driven by membrane potential, our use of PINK1-CA revealed that PINK1 activation rather than mitochondrial dysfunction was sufficient to induce cell death.

Caspases are cysteine-aspartic proteases with critical roles in the regulation of apoptosis and are essential for various cellular events (35). We demonstrated that cell death induced by PINK1 activation was not affected by treatment with a pan-caspase inhibitor and that caspase-3 and PARP processing were barely detected in HA-PARKIN-expressing HeLa cells upon CCCP treatment.

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**FIGURE 5.** PARKIN ubiquitin ligase activity and TOM20 are required for PINK1-CA-induced cell death. A, HeLa cells transfected with GFP or the indicated GFP-PARKIN mutant, together with PINK1(ΔN34), were cultured for 48 h and stained with PI. B, HA-PARKIN-expressing HeLa cells were transfected with siRNA for Luc or TOM20. After 48 h, cells were further transfected with or without plasmid carrying PINK1(ΔN34) or PINK1(ΔN34, KD), cultured for 30 h, and stained with PI. C, HeLa cells were transfected with siRNA for either Luc or TOM20, and total cell lysates were analyzed by immunoblot with antibodies to TOM20 or tubulin as a loading control. D, HeLa cells transfected with siRNA for Luc or TOM20 were cultured for 48 h, and cells were further transfected with PINK1(ΔN34)-3HA. Targeting of PINK1(ΔN34) to the mitochondria was analyzed by immunofluorescence microscopy. E, HA-PARKIN-expressing cells were co-transfected with CFP and plasmid carrying either PINK1(ΔN34) or PINK1(ΔN34, KD), cultured for 6 h, further incubated for 24 h with either DMSO, 2 μm necrosulfonamide (NSA), or 10 μM necrostatin-1 (Nec-1), and stained with PI. F, HA-PARKIN-expressing cells were transfected with siRNA for either Luc or MLKL, cultured for 48 h, further co-transfected with GFP and plasmid carrying either PINK1(ΔN34) or PINK1(ΔN34, KD), additionally incubated for 30 h, and stained with PI. G, HeLa cells were transfected with a siRNA for either Luc or MLKL, and total cell lysates were analyzed by immunoblot with antibodies to MLKL or p97 as a loading control. Data in A and C–F represent the mean ± S.E. of three independent experiments (>100 individual cells were counted). *, p < 0.05; **, p < 0.005; ***, p < 0.0005 (Student’s t test). n.s. represents not significant.
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FIGURE 6. Proteasome activity, but not autophagy, is necessary for PINK1-mediated cell death. A, HA-PARKIN-expressing cells were co-transfected with GFP and plasmid carrying PINK1(ΔN34) or PINK1(ΔN34, KD), cultured for 12 h, further incubated for 18 h with DMSO, 5 μM lactacystin, or 0.1 μM bortezomib, and stained with PI. B, HEK293 cells were co-transfected with GFP and plasmid carrying PINK1(ΔN34) or PINK1(ΔN34, KD), cultured for 24 h, further incubated for 24 h with DMSO or 5 μM lactacystin, and stained with PI. C, HA-PARKIN-expressing cells were treated as described in A and stained with Zombie Green. D, HA-PARKIN-expressing cells were co-transfected with plasmid carrying PINK1(ΔN34) or PINK1(ΔN34, KD) and cultured for 48 h. Proteasome activities were measured as described under "Experimental Procedures." E, control HeLa (WT), FIP200-knock-out, and ATG7-knock-out cell lines were transfected with mito-Keima and cultured for 24 h under normoxic (panels a, c, and e) or hypoxic conditions (panels b, d, and f). Live images were obtained using fluorescence microscopy. Green and red signals represent mito-Keima in neutral and acidic pH environments, respectively. Scale bar, 10 mm. F, control HeLa (WT), FIP200-knock-out, and ATG7-knock-out cell lines were transfected with either GFP-PARKIN (panels a–l) or GFP (panels m–x), together with plasmid carrying PINK1(ΔN34), and cultured for 48 h. Live images were obtained using fluorescence microscopy. Fluorescent signals of mito-Keima in neutral and acidic pH environments were detected by excitation using 430 and 560 nm of light, respectively. Scale bar, 10 mm. G, FIP200-knock-out (clones 13 and 15) and ATG7-knock-out (clones 13 and 23) HeLa cell lines were co-transfected with GFP-PARKIN and plasmid carrying PINK1(ΔN34) or PINK1(ΔN34, KD), cultured for 48 h, and stained with PI. H, left panels: HA-PARKIN-expressing HeLa cells were co-transfected with CFP and plasmids carrying PINK1(ΔN34) or PINK1(ΔN34, KD) and cultured for 30 h. All cells were co-stained with PI and MitoTracker Green, which specifically accumulates into mitochondria irrespective of their membrane potential. Live images of the treated (panels a–d) and transfected cells (panels e–h) were obtained by fluorescence microscopy using a fluorescence optical sectioning system. Scale bar, 10 μm. Data in A–D and G represent the mean ± S.E. of three independent experiments (>100 individual cells were counted). *, p < 0.05; **, p < 0.005; ***, p < 0.0005 (Student’s t test). n.s. represents not significant.

treatment. Furthermore, electron microscopic analysis revealed that none of the cells had the characteristic morphology of apoptotic cells, including nuclear fragmentation, chromatin condensation, and cell shrinkage, even without the pan-caspase inhibitor. Thus, the cell death induced by PINK1 activation was not a typical apoptotic event. Recent studies, however, showed that PARKIN plays a critical role in apoptotic death in cooperation with PINK1 (36, 37). The possibility that multiple pathways of cell death contribute to achievement of PINK1-mediated cell killing cannot be ruled out, however, because inhibiting caspases shifts cell fate to non-apoptotic pathways (38).
motic activity of PARKIN, suggesting that ubiquitination of the outer membrane proteins is necessary to kill cells. These processes toward ubiquitination were the same as for selective autophagy of mitochondria. PINK1-mediated cell death, however, was unaffected by knocking out the FIP200 and ATG7 genes, which code for components of the autophagic machinery (39, 40). Thus, the PINK1-mediated cell death was independent of selective autophagy for mitochondria, although mitochondrial autophagy occurred in advance of the cell death. This finding was supported by the discovery of mitochondria in dead cells by fluorescence and electron microscopic analyses. Furthermore, the observation of mitochondria in the dead cells clearly demonstrated that the PINK1-mediated cell death was not due to a complete loss of mitochondria. Conversely, the remaining damaged mitochondria may lead to the propagation of cell death.

In contrast to autophagy, proteasomal activity was indispensable for the cell death induced by PINK1 activation, suggesting that rupture of the outer membrane of the mitochondria is associated with cell death. In addition to the outer membrane, some of the mitochondrial inner membrane proteins are degraded by proteasomes in a PARKIN-dependent manner (33). Therefore, the integrity of both the outer and inner membranes may be impaired by proteasomal degradation when PINK1 is activated. Such damaged mitochondria are thought to easily release non-proteinaceous contents, such as iron, into the cytoplasm. The potential release of small molecules, however, is distinct from MPT because MPT was dispensable for the cell death induced by PINK1 activation. As a consequence of the mitochondrial membrane rupture, the production of toxic compounds or a homeostatic imbalance in the cytoplasm may occur, and cells will eventually die. Another type of non-apoptotic cell death, termed ferroptosis, is an iron-dependent form that involves the loss of cytosolic glutathiones and the subsequent accumulation of ROS in the cell (10, 41). Actually, ROS elevation prior to the cell death was detected in PARKIN-expressing HeLa cells. Taken together with the finding of some mitochondria in dead cells, PINK1-mediated cell death is a distinct type of cell death that requires proteasomes and may provide a way to eliminate cells that fail to rid themselves of all damaged mitochondria.

The unfolded protein response is a well characterized cellular process that is governed by three signaling pathways and switches between life and death fates for the cell by differences in the duration of endoplasmic reticulum stress (42). Such cell protective and cell death responses have the common purpose of avoiding the accumulation of misfolded proteins in cells and tissues. Similar to misfolded proteins, damaged mitochondria might be eliminated to reduce ROS production. For complete elimination of damaged mitochondria, PINK1 initially induces autophagy-mediated selective clearance to maintain mitochondrial quality, and may eventually trigger proteasome-dependent suicide of cells in which damaged mitochondria are still retained. Further studies are needed to elucidate the molecular mechanism of programmed cell death in association with PINK1 activation.

### Experimental Procedures

**Cell Lines**—HeLa cell lines stably expressing GFP-PARKIN, HA-PARKIN, or Bcl-XL were established previously (6, 19). HEK293, SH-SY5Y, the above HeLa stable cell lines, and ATG7- and FIP200-knock-out HeLa cell lines were maintained at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 4.5 mg/ml glucose.

**Reagents and Antibodies**—The following reagents were used: propidium iodide (PI, Dojin Chemicals); carbonyl cyanide m-chlorophenylhydrazine (CCCP), A23187, actinomycin D, and cyclosporine A (Sigma); Z-VAD-fmk, MG132, and lactacystin (Peptide Institute Inc.); bongkrekic acid (Abcam); Phos-tag acrylamide (Wako Chemicals); necrosulfonamide (Cellagen Technology); necrostatin-1 (Focus Biomolecules); bortezomib (Selleck Chemicals); MitoTracker Green (Life Technologies, Inc.); tetramethylrhodamine methyl ester perchlorate (Invitrogen); Zombie Green (BioLegend).

The following primary antibodies were used for immunoblotting: PARP (clone 42/PARP) and caspase-3 (clone 46/caspase-3; BD Biosciences); cleaved caspase-3 (catalog no. 9661, Cell Signaling); p97 (clone 58.13.3, PROGEN Biotechnik); PINK1 (BC100–494, Novus); mitofusin 2 (clone 4H8, Sigma); HA (clone 4B2, Wako Chemicals); and Mcl-1 (clone S-19) and parkin (clone PRK8, Santa Cruz Biotechnology). Antibodies against the HA tag (clone 3F10, Roche Applied Science), TOM20 (FL-145, Santa Cruz Biotechnology), mtHSP70 (clone JG1, Thermo Scientific), cytochrome c (clone 6H2.B4, BD Bioscience), or mitofillin (43) were used for immunofluorescence microscopy.

**DNA and siRNA Transfection**—The plasmids used in this study are summarized in Table 1. DNA transfection was performed using FuGENE HD (Promega) according to the manufacturer’s instructions. The following siRNA duplexes were transfected into HeLa cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions: PINK1 (target sequence, gagcguuccgcgguaaga) and TOM20 (target sequence, aagattacctgaccttaaga). After incubation for 48 h, the cells were harvested and used for immunoblotting.

### Table 1: Plasmids used in this study

<table>
<thead>
<tr>
<th>Vector</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
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<tr>
<td>pEGFP-C1-parkin</td>
<td>For expression of GFP-PARKIN</td>
<td>6</td>
</tr>
<tr>
<td>pEGFP-C1-parkin (T415N)</td>
<td>For expression of GFP-PARKIN (T415N)</td>
<td>6</td>
</tr>
<tr>
<td>pEGFP-C1-parkin (G430D)</td>
<td>For expression of GFP-PARKIN (G430D)</td>
<td>6</td>
</tr>
<tr>
<td>pCMVNT(d1)-PINK1(ΔN34)-3HA</td>
<td>For weak expression of PINK1 (ΔN34)-3HA</td>
<td>13</td>
</tr>
<tr>
<td>pCMVNT(d1)-PINK1(ΔN34, KD)-3HA</td>
<td>For weak expression of PINK1 (ΔN34, KD)-3HA</td>
<td>This study</td>
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<td>pCMVNT(d1)-PINK1(ΔN34, S402A)-3HA</td>
<td>For weak expression of PINK1 (ΔN34, S402A)-3HA</td>
<td>This study</td>
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**Immunoblotting**—Immunoblotting was performed essentially as described previously (44). Phosphorylated proteins were detected on acrylamide gels with 50 \( \mu \)M Phos-tag acrylamide (Wako Chemicals), and 100 \( \mu \)M MnCl\(_2\) was used. After electrophoresis, the gels were incubated for 10 min with the buffer (20% methanol, 25 mM Tris, 192 mM glycine, and 0.01% SDS) containing 1 mM EDTA and then washed for 10 min using the same buffer without EDTA. The proteins were transferred to PVDF membranes (Merck Millipore) and immunoblotted with the indicated antibodies using the Immun-Star AP Chemiluminescence kit (Bio-Rad) according to the manufacturer’s instructions.

**Immunofluorescence Microscopy**—Immunofluorescence microscopy was performed essentially as described previously (44), with the following modification: cells grown on glass coverslips were fixed at room temperature for 25 min with 4% paraformaldehyde, permeabilized by incubation with 0.1% Triton X-100 for 10 min, and incubated with the indicated primary antibodies. Images were acquired using a confocal laser-scanning microscope LSM510 (Carl Zeiss Inc.).

**PI Staining**—Plasma membrane integrity based on the exclusion of PI was measured as an index of dead cells. Cells were treated with 10 \( \mu \)M CCCP for the indicated times before staining with PI. To induce cell death by introducing PINK1(ΔN34), the plasmid carrying PINK1(ΔN34)-3HA was transfected together with a plasmid containing either GFP or cyan fluorescent protein (CFP) as a marker. In addition to the cells attached to culture dishes, the cells floating in the culture medium were collected, and all harvested cells were resuspended in phosphate-buffered saline (PBS). Cells were stained at 37 °C for 15 min with PI (1 \( \mu \)M final concentration). After incubation, stained cells were scored as dead cells under a fluorescence microscope.

To acquire live fluorescence images, cells were grown on glass bottom dishes (Greiner Bio One) coated with poly-L-lysine (Sigma). After washing with PBS, the cells were stained for 15 min with 1 \( \mu \)M PI in PBS. Without fixation, live fluorescence images were obtained by fluorescence microscopy using a fluorescence optical sectioning system VivaTome (Carl Zeiss).

**Vital Staining with Zombie Green**—Vital staining with Zombie Green was performed essentially as described for the PI staining with the following modification: cells were stained at room temperature for 20 min with Zombie Green. After incubation, stained cells were washed with PBS containing 1% bovine serum albumin and scored as dead cells under a fluorescence microscope.

**Luminescence Cytotoxicity Assay**—According to the manufacturer’s instructions, the relative number of dead cells in the populations was determined using a luminescence cytotoxicity assay kit (CytoTox-Glo, Promega) that measured the activities of proteases released from impaired cells.

**Measurement of Intracellular ATP**—ATP levels of the cell populations were quantified using a luminescence assay kit (CellTiter-Glo Luminescent Cell Viability Assay, Promega), according to the manufacturer’s instructions. The resultant luminescent units were converted using ATP solution (New England Biolabs) as a standard.

**Measurement of ROS Production**—Generation of ROS in the cultured cells was measured using a luminescence assay kit (ROS-Glo H\(_2\)O\(_2\) Assay, Promega), according to the manufacturer’s instructions.

**Determination of Proteasome Activity**—Proteasome activity in the cell populations was determined using a luminescence assay kit (Proteasome-Glo chymotrypsin-like cell-based assay, Promega), according to the manufacturer’s instructions.

**Construction of FIP200 and ATG7 KO by CRISPER**—ATG7-KO and FIP200-KO HEla cells were generated using the CRISPR/Cas9 system (45). Two independent guide RNAs were selected from each ATG7 exon 2 (GGCGAGCCTACGCGGGATCCTGG and GAAGCTGAAACGATCTCGGCTGG) and FIP200 exon 4 (CACCAAGTGCTGTGTAATGG and GTGTACCTACAGTGTGGGACGG). These target oligonucleotides were ligated into pX330-U6-Chimeric_BB-BCBh-hSpCas9, a gift from Feng Zhang (Addgene plasmid 42230). The resultant plasmid was co-transfected with PBS-Puro containing a puromycin-resistant gene as the selection marker. One day after transfection, the transfected cells were enriched by incubation with medium containing puromycin and then cloned by limiting dilution in 96-well plates. The KO cell lines were verified by immunoblot analysis with anti-ATG7 or anti-FIP200 antibodies and DNA sequence analysis of the target regions.

**Measurement of Mitochondrial Autophagy**—To determine the delivery of mitochondria to the acidic compartments, including lysosomes, we utilized the mitochondria-targeted and pH-sensitive fluorescent protein, mito-Keima (17). The pH sensitivity of mito-Keima is a powerful tool to determine whether mitochondria were delivered to acidic environments. When present in neutral conditions (e.g., mitochondrial matrix), mito-Keima is excited by 430 nm of light, but not by 560 nm of light. In acidic conditions, including lysosome, however, the fluorescent protein is excited by 560 nm of light, but not by 430 nm of light. HeLa cells expressing HA-PARKIN were transfected with mito-Keima (pMT-mKeima-Red, MBL) and cultured in the presence of CCCP (10 \( \mu \)M). Fluorescence images were acquired using a 60× oil immersion objective on a fluorescence microscope (IX73, Olympus). To observe mito-Keima, two excitation filters (430/24 nm for the neutral condition and 560/40 nm for the acidic condition) and an emission filter (630/60 nm) were applied. Puncta of mito-Keima in the acidic condition were quantified using Metamorph version 7.8 (Molecular Devices). Cells with more than 15 fluorescent dots of mito-Keima excited by 560 nm of light were scored as an index of mitochondrial autophagy.

**Electron Microscopy**—Samples were incubated with 2% paraformaldehyde and 2% glutaraldehyde, further fixed with 0.5% tannic acid, dehydrated, and subjected to embedding. Ultrathin sections were stained with 2% uranyl acetate, and images were obtained using a transmission electron microscope (JEM-1400Plus; JEOL Ltd., Tokyo, Japan) with a CCD camera (VELETA; Olympus Soft Imaging Solutions GmbH, Munich, Germany).

**Statistical Analysis**—Statistical analysis was performed using a one-tailed or two-tailed Student’s t test. A \( p \) value of less than 0.05 was considered statistically significant.
Autophagy and requires the MAPK1 and MAPK14 signaling pathways. Autophagy 11, 332–343.


