ORIGINAL RESEARCH COMMUNICATION

Parkin Regulates Programmed Necrosis and Myocardial Ischemia/Reperfusion Injury by Targeting Cyclophilin-D

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Abstract

Aims: Cardiomyocyte death critically contributes to the pathogenesis of cardiac disorders, such as myocardial infarction, heart failure, and cardiac ischemia/reperfusion (I/R) injury. As one of the main forms of cardiac cell death, necrosis plays a critical role in heart diseases. Multiple signaling pathways of necrosis have been demonstrated, in which death receptors, receptor-interacting serine/threonine-protein 1 and 3 kinases, and cyclophilin-D (CypD) have been deeply implicated. However, the fundamental mechanism underlying myocardial necroptosis, especially the mitochondrial permeability transition pore (mPTP)-CypD-dependent death pathway, is poorly understood. Parkin functions as an E3 ubiquitin protein ligase that mainly mediates mitophagy cascades. As yet, it is not clear whether Parkin participates in regulating necrosis and myocardial I/R injury.

Results: Here, our results showed that Parkin mediated mitophagy and inhibited necrosis under oxidative stress. In further exploring the underlying mechanisms, we found that Parkin suppressed mPTP opening by catalyzing the ubiquitination of CypD in necrotic cascades, which were not involved in Parkin-regulated mitophagy. Parkin inhibited necrosis, reduced myocardial I/R injury, and improved cardiac function.

Innovation: Our present work reveals a highlighted connection between the mitochondrial matrix-localized Parkin and the mPTP-CypD-dependent necrotic signaling pathway in cardiac injury.

Conclusion: Our results revealed a novel myocardial necrotic regulating model composed of Parkin, CypD, and mPTP, which may provide potential therapeutic targets and strategies to modulate the levels of these molecules. *Antioxid. Redox Signal.* 31, 1177–1193.

Keywords: Parkin, CypD, mPTP, programmed necrosis, myocardial ischemia/reperfusion injury

Introduction

CARDIOVASCULAR DISEASES REMAIN the top major killers worldwide (2). Several studies have demonstrated that cell death contributes greatly to the pathogenesis of various heart diseases, including myocardial infarction (MI), ischemia/reperfusion (I/R), and heart failure (HF) (1, 17). Cardiac cell death has been classified into different forms, including necrosis, apoptosis, and autophagy, based on cell morphology and biochemical features (16). Apoptosis is an active and tightly regulated process, whereas necrosis has long been considered an accidental or passive type of cell death (16). Over the past three decades, studies have mainly focused on

apoptotic cell death in the heart as the only form of regulated cell death amenable to experimental manipulation. However, accumulated evidence has shown that necrosis is the major type of cell death in severe myocardial damage (1, 17, 62), indicating its significance in the pathological process of cardiac diseases.

Recent advances have revealed the existence of multiple pathways of regulated necrosis, termed “programmed necrosis” or “necroptosis.” The receptor-dependent way and mitochondrial way are the two major ways involved in myocardial necrosis (29, 38, 50). Receptor-interacting (RIP) 1 and 3 kinases were activated in ischemic hearts, and these enzymes mediate myocardial necroptosis (30, 42, 54, 62).

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Innovation

The significance and multiple pathways of necroptosis in heart diseases have been demonstrated. However, the fundamental mechanism involving the mitochondrial permeability transition pore (mPTP)-cyclophilin-D (CypD)-dependent death pathway underlying myocardial necroptosis is poorly understood. Our present work highlighted the connection between mitochondrial matrix-localized Parkin and the mPTP-CypD-dependent necrotic signaling pathway in cardiac injury. We discovered that CypD was a novel substrate for Parkin ubiquitination, which contributed to the protective activity of Parkin against necroptosis in cardiac injury. The modulation of Parkin may provide potential therapeutic targets and strategies to treat myocardial necrosis-referred heart diseases, including myocardial infarction and heart failure.

Necrostatin-1, a pharmacological inhibitor of RIP1 kinase, can inhibit myocardial cell death and reduce infarct size in models of cardiac infarction (42). A recent study found that RIP3 regulated necroptosis by targeting Ca^{2+} -calmodulin-dependent protein kinase II in myocardial I/R damage (62). Our previous study showed that Fas-associated protein, with a death domain, regulates I/R and oxidative stress-induced necrotic cardiomyocyte death by influencing the receptor-interacting serine/threonine-protein kinase (RIPK)/RIPK3 complex (54). Another important necrosis mechanism involves mitochondrial permeability transition pore (mPTP) opening, which is regulated by cyclophilin-D (CypD) (27, 50). However, the fundamental mechanism underlying myocardial necroptosis, especially the mPTP-CypD-dependent death pathway, is poorly understood.

Mitochondria dysfunction contributes to programmed necrosis initiation by regulating mPTP (38). mPTP, which spans the inner mitochondrial membrane (IMM) and outer mitochondrial membrane (OMM), respectively, is a non-specific channel that plays a critical role in mitochondrial morphological and functional regulation (22). The mPTP opens or closes to control the solute exchange between the mitochondrial matrix (MA) and the surrounding cytoplasm. However, the prolonged opening of the mPTP leads to the loss of IMM potential, followed by mitochondrial energetic dysfunction, organelle swelling, rupture, and, eventually, necrotic cell death, which contributes to the pathogenesis of many diseases, including myocardial injury (26, 38, 46).

CypD protein, a peptidyl-prolyl isomerase located in the mitochondrial MA, is a key component of the mPTP complex and it is critically involved in regulating mPTP opening (46). CypD-overexpressing mice demonstrated swollen mitochondria, which makes these animals more vulnerable to cell death (38). CypD deficiency protected cardiomyocytes against I/R injury and enhanced resistance to necrotic cell death but not apoptosis (38), thereby suggesting a critical role for CypD in necrosis. CypD knockout mice have higher rates of glutamate/malate-dependent oxidative phosphorylation, higher membrane potential, and higher resistance to detrimental Ca^{2+} effects than those of wild-type mice (9, 35, 38). In addition, cyclosporine A potently prevents mPTP opening by binding to CypD, which reduces myocardial injury as a therapeutic strategy (13). Direct interaction with p53 (50),

S-nitrosylation (41), and deacetylation by Sirt3 (18) modulated the activity of CypD. However, the regulation of CypD in necrosis is largely unknown.

Parkin (*PARK2*) was first identified as a Parkinson's disease (PD)-associated gene (23) and functions as an E3 ubiquitin-protein ligase involved in the ubiquitin-proteasome dependent pathway (47). By preventing mitochondrial swelling and cytochrome *c* release, Parkin protects against dopaminergic cell death (6). Loss of Parkin function results in dysfunctional mitochondria and increased mitochondrial fragmentation (31). The elimination of damaged mitochondria is processed by Parkin-mediated autophagy. The Pink1-dependent selective recruitment of Parkin to impaired mitochondria is essential for mitophagy (52).

There is evidence that Parkin is highly expressed in the heart and contributes significantly to heart development (14). Parkin directs cardiac metabolic maturation and mitochondria removal during the perinatal period. Parkin deficiency inhibits postnatal mitochondrial maturation that is critical to survival (14). Parkin levels were downregulated on cardiac disorders such as I/R injury (24), hypoxia injury (44), diabetic cardiomyopathy (57), myocardial aging, and cardiac hypertrophy (58). Parkin reduction participates in cardiac pathological process, but the underlying mechanism is not known (20, 23, 24). Thus, we were interested in whether Parkin participates in oxidative stress-induced necrosis in cardiomyocytes.

Our present work aimed at exploring the function of Parkin in necrotic cell death. Parkin was found to regulate necrotic cell death by preventing the opening of the mPTP in cardiomyocytes. We also discovered that CypD is a novel substrate for Parkin ubiquitination, which contributed to the protective activity of Parkin against necrosis in cardiac injury. Taken together, we reveal a novel necrosis regulatory model comprising Parkin and CypD.

Results

Parkin translocates to mitochondria and is downregulated under oxidative stress

There is evidence that Parkin is highly expressed in the heart and is involved in the pathogenesis of cardiac disorders (20, 23). To understand the role of Parkin in oxidative stress-induced cell death in cardiomyocytes, we measured its expression in neonatal rat cardiomyocytes exposed to different concentrations of H_2O_2 . Both the protein and mRNA levels of Parkin were downregulated in a dose-dependent manner (Fig. 1A, B). The level of Parkin was substantially downregulated in cardiomyocytes exposed to $500 \mu\text{M}$ H_2O_2 , but it was clearly unchanged in cardiomyocytes exposed to $100 \mu\text{M}$ H_2O_2 (Fig. 1A, B). Parkin is recruited to damaged mitochondria in response to stimuli (40, 60). Oxidative stress can induce mitochondrial damage, suggesting that Parkin might translocate to mitochondria in cardiomyocytes under oxidative stress. We also detected the subcellular location of Parkin in cardiomyocytes on 50 – $750 \mu\text{M}$ H_2O_2 treatment. The levels of Parkin in mitochondria-enriched heavy membranes were increased, peaking after $100 \mu\text{M}$ H_2O_2 treatment and then decreasing with increasing H_2O_2 concentration because of the downregulation of Parkin (Fig. 1A).

A mouse I/R model is usually used for studying MI. I/R has been demonstrated to induce reactive oxygen species

production in the heart. Both the protein and mRNA levels of Parkin were downregulated in response to I/R injury in the ischemic zone but not in the border zone of the heart (Fig. 1C, D). Regarding the subcellular location of Parkin in the heart, its levels were elevated in mitochondria at the border zone but not at the ischemic zone in response to I/R injury (Fig. 1C).

To further confirm the intracellular localization of Parkin, immunofluorescence was used to monitor its translocation in cardiomyocytes. In the control group of untreated cells, Parkin was distributed throughout the cytoplasm and showed a distinct pattern by MitoTracker (Fig. 1E). On 100 μM H_2O_2 treatment, Parkin accumulated around the perinuclear region, and its pattern was very similar to that of MitoTracker (Fig. 1E). On 500 μM H_2O_2 treatment, Parkin was nearly undetectable (Fig. 1E). Taken together, these data suggest that Parkin translocates to mitochondria on low-dose oxidative stress but is downregulated on high-dose oxidative stress. The function of Parkin may be altered under different severities of pathological conditions.

The intramitochondrial localization of endogenous Parkin was also confirmed by some studies (25, 45). We further analyzed the submitochondrial localization of Parkin on

100 μM H_2O_2 treatment. The results confirmed that a fraction of Parkin accumulated on the surface (outer membrane [OM]), whereas another portion was localized to the inner membrane (IM) and MA of mitochondria in cardiomyocytes (Supplementary Fig. S1A). Moreover, the mitochondrial uncoupling agent carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was used to induce mitochondrial damage, on which Parkin translocates to mitochondria (Supplementary Fig. S1B). Consistent with the results of oxidative stress, Parkin was detected in the intramitochondrial fraction of cardiomyocytes exposed to CCCP (Supplementary Fig. S1C).

Parkin participates in H_2O_2 -induced mitophagy in cardiomyocytes

The importance of Parkin in mitophagy has been reported by many works (3, 33, 40). However, whether Parkin participates in H_2O_2 -induced mitophagy in cardiomyocytes is unclear. Light chain 3 (LC3) protein is necessary for the execution of mitophagy and has been widely applied as an autophagosome marker (37). We used LC3-green fluorescent protein (GFP) to indicate mitophagy initiation. Immunofluorescence analysis showed that low-dose H_2O_2 caused LC3-GFP accumulation around the perinuclear region and LC3-GFP colocalization with mitochondria, which was disrupted by increasing the concentration of H_2O_2 (Fig. 2A). The conversion of LC3-I to LC3-II refers to autophagic activity, and the amount of LC3-II relates well with the number of autophagosomes (37). A significant increase in LC3-II/LC3I and autophagic vacuoles formation in cardiomyocytes treated with low-dose H_2O_2 were detected (Supplementary Fig. S2A, B). Thus, these results indicate that mitophagy in cardiomyocytes is induced by a low dose of H_2O_2 but not by a high dose of H_2O_2 .

FIG. 1. Parkin is downregulated and transfers to mitochondria in H_2O_2 -treated cardiomyocytes and ischemic heart. (A) Protein expression levels of Parkin were detected in whole-cell lysates or mitochondria-enriched HMs or cytosol of cardiomyocytes exposed to H_2O_2 for 6 h at the indicated concentration. $n=3$ experiments per group. (B) Parkin mRNA levels were analyzed in cardiomyocytes exposed to H_2O_2 for 6 h at the indicated concentration. $*p<0.05$ versus control. $n=3$ experiments per group. (C) Detection of Parkin expression levels in myocardium subjected to ischemia injury. Mice were subjected to cardiac ischemia at the indicated time. The protein levels of expression levels of Parkin protein were detected by immunoblot in whole-cell lysates or HMs of ischemia zone and border zone, respectively. $n=6$ mice per group. (D) Expression levels of Parkin mRNA were detected in ischemia zone and border zone from hearts subjected to ischemia injury at the indicated time. $*p<0.05$ versus sham. $n=7$ mice per group. (E) Subcellular localization analysis of Parkin in cardiomyocytes. Cardiomyocytes were treated with 100 or 500 μM H_2O_2 for 6 h. Distribution of Parkin was detected by immunofluorescence. Cardiomyocytes were labeled with MitoTracker (red), stained with anti-Parkin antibody, and monitored by FITC-labeled secondary antibody (green) and DAPI (blue). Bar=10 μm . Data are expressed as mean $\pm$ SD. $n=3$ experiments per group. DAPI, 4',6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; HM, heavy membrane; SD, standard deviation. Color images are available online.

FIG. 2. Parkin participates in H₂O₂-induced mitophagy in cardiomyocytes. (A) Detection of LC3 expression pattern in cardiomyocytes exposed to H₂O₂. Cardiomyocytes were treated with H₂O₂ for 6 h at the indicated concentration after being transfected with GFP-LC3 plasmid. GFP-LC3 expression patterns are shown in *green*. Mitochondria was stained by MitoTracker, shown in *red*. Nuclei were stained by DAPI, shown in *blue*. Representative images are shown on the *left*; bar=10 μ m. GFP-LC3 puncta-positive cells were quantified (*right*); * p <0.05 versus control. n =4 experiments per group. (B–D) Overexpression of Parkin increased the expression level of LC3-II and the ratio of LC3II/LC3I (B), the number of autophagic vacuoles (C), and the number of GFP-LC3 puncta-positive cells (D) in cardiomyocytes treated with 500 μ M H₂O₂. The protein levels of LC3 and Parkin were detected by immunoblot. * p <0.05 versus H₂O₂ alone. n =3 experiments per group. The autophagosomes were analyzed by electron microscopy. Bar=2 μ m. The *arrows* depict autophagosomes, and the nucleus is denoted by N. Quantification of autophagic vacuoles is shown in the *right panel*. * p <0.05, n =5 experiments per group. (E, F) Knockdown of Parkin by its siRNA reduced the number of GFP-LC3 puncta-positive cells (E) and the expression level of LC3-II (F) in cardiomyocytes exposed to 100 μ M H₂O₂. * p <0.05 versus H₂O₂ alone. Data are expressed as mean $\pm$ SD, n =3 experiments per group. GFP, green fluorescent protein; LC3, light chain 3. Color images are available online.

Next, we tested whether Parkin participates in H_2O_2 -induced mitophagy in cardiomyocytes. Besides LC3, Atg5 and Atg12 were used as autophagy markers. The enforced expression of Parkin triggered mitophagy on high-dose H_2O_2 treatment (Fig. 2B–D and Supplementary Fig. S2C), whereas the knockdown of Parkin attenuated the mitophagy induced by low-dose H_2O_2 (Fig. 2E, F and Supplementary Fig. S2D). Therefore, we concluded that Parkin regulates H_2O_2 -induced mitophagy in cardiomyocytes.

Parkin protects against H_2O_2 -induced mPTP opening and necrotic cell death

The form of cell death induced by H_2O_2 varies from apoptosis to necrosis, depending on the exact concentration applied and the cell type tested (54). Our previous work indicated that apoptosis was mainly triggered by $100 \mu M$ H_2O_2 , whereas necrosis was induced by $500 \mu M$ H_2O_2 in cardiomyocytes (54). Parkin was downregulated on $500 \mu M$ H_2O_2 treatment (Fig. 1A, B), which indicates that this protein may

be involved in H_2O_2 -induced necrotic cell death. The mPTP opened under oxidative stress, and mPTP opening is considered a typical event of necrosis (62). Therefore, we first tested whether mPTP opening was affected by different concentrations of H_2O_2 . The opening of mPTP resulted in the dissipation of the mitochondrial membrane potential ($\Delta\Psi_m$) across the IMM, which could be assessed by the cationic fluorescent dye tetramethylrhodamine methylester (TMRM) (59). H_2O_2 -induced mPTP opening depends on the concentration of H_2O_2 . In response to $500 \mu M$ H_2O_2 treatment, TMRM fluorescence was completely lost in cardiomyocytes (Fig. 3A). The enforced expression of Parkin attenuated high-dose H_2O_2 -induced mPTP opening (Fig. 3B). Low-dose H_2O_2 slightly induced the loss of TMRM fluorescence, whereas Parkin knockdown strengthened the mPTP opening in cardiomyocytes exposed to $100 \mu M$ H_2O_2 (Fig. 3C). Taken together, these results indicate that Parkin could inhibit H_2O_2 -induced mPTP opening.

Next, we studied the function of Parkin in cardiomyocyte necrosis. We first tested the effect of different concentrations

FIG. 3. Parkin can inhibit H_2O_2 -induced mPTP opening and cell death in cardiomyocytes. (A) mPTP opening was assessed. Cardiomyocytes were treated with 100 or $500 \mu M$ H_2O_2 , then stained by TMRM. Fluorescence intensity was detected at the indicated time. $n=3$ experiments per group. (B) Overexpression of Parkin reduced the fluorescence intensity of TMRM in $500 \mu M$ H_2O_2 -treated cardiomyocytes. $*p<0.05$ versus H_2O_2 alone. $n=3$ experiments per group. (C) Knockdown of Parkin increased the fluorescence intensity of TMRM in $100 \mu M$ H_2O_2 -treated cardiomyocytes. $*p<0.05$ versus H_2O_2 alone. $n=3$ experiments per group. (D, E) Overexpression of Parkin inhibited H_2O_2 -induced necrotic cell death in cardiomyocytes treated with $500 \mu M$ H_2O_2 for 24 h. Necrosis was detected by PI assay (D) and LDH activity assay (E). $*p<0.05$ versus H_2O_2 alone. $n=3$ experiments per group. (F, G) Knockdown of Parkin by its siRNA strengthened H_2O_2 -induced necrotic cell death in cardiomyocytes treated with $100 \mu M$ H_2O_2 for 24 h. $*p<0.05$ versus H_2O_2 alone. Data are expressed as mean $\pm$ SD, $n=3$ experiments per group. LDH, lactate dehydrogenase; mPTP, mitochondrial permeability transition pore; PI, propidium iodide; TMRM, tetramethylrhodamine methylester. Color images are available online.

of H_2O_2 on cardiomyocyte death and observed that treatment with $100\ \mu\text{M}$ H_2O_2 mainly triggered apoptosis, whereas $500\ \mu\text{M}$ H_2O_2 treatment preferentially caused necrosis (Supplementary Fig. S3A, B). This result was further confirmed by transmission electron microscopy (Supplementary Fig. S3C). The overexpression of Parkin attenuated the necrosis induced by $500\ \mu\text{M}$ H_2O_2 (Fig. 3D, E). The knockdown of endogenous Parkin sensitized cardiomyocytes to necrosis (Fig. 3F, G). In addition, H_2O_2 -induced apoptosis was not influenced by the change in Parkin expression (Supplementary Fig. S3D).

Tumor necrosis factor- α (TNF- α) combination with apoptosis inhibitor carbobenzoxy-valylalanyl-aspartyl-[*O*-methyl]-fluoromethylketone (z-VAD-fmk) has been demonstrated to induce RIP1/RIP3-dependent necrotic cell death in many types of cells such as cardiomyocytes (5, 19, 54). Parkin participated in H_2O_2 -induced necrosis, which led us to consider whether it is involved in TNF- α -induced necrosis. The results showed that necrotic cell death was not affected by overexpression of Parkin in cardiomyocytes combinatorially treated with TNF- α and Z-VAD (Supplementary Fig. S3E). Therefore, we concluded that Parkin is not involved in TNF- α -induced necrosis.

Parkin interacts with CypD in cardiomyocytes under oxidative stress

CypD in the mitochondrial MA is an essential regulator of mPTP opening. Parkin accumulates in mitochondria and inhibits H_2O_2 -induced mPTP opening. Thus, we examined whether Parkin inhibits necrosis by interacting with CypD in cardiomyocytes treated with H_2O_2 . First, we tested the influence of Parkin on the expression level of CypD. The protein levels of CypD were not changed in cardiomyocytes exposed to H_2O_2 (Fig. 4A). Neither enforced expression nor knockdown of Parkin affected CypD expression (Fig. 4B, C).

We next tested whether Parkin binds to CypD directly. HEK-293 cells were transfected with plasmids expressing Myc-CypD together with GFP-Parkin-vector or mito-GFP-Parkin-vector (Parkin fused to mitochondria-targeted GFP) or mito-GFP-vector (mitochondrial-targeted GFP). Cell lysates were then analyzed by immunoprecipitation with an anti-GFP antibody. As shown in Figure 4D, Myc-CypD was immunoprecipitated by GFP-Parkin and mito-GFP-Parkin, especially mitochondria-localized Parkin. Next, we investigated whether endogenous CypD interacts with endogenous Parkin. Cardiomyocytes were overexpressed with Parkin, and the cell lysates were subjected to immunoprecipitation with an anti-Parkin antibody or immunoglobulin G control, followed by Western blotting. The enforced expression of Parkin led to a significant increase in immunoprecipitated CypD (Fig. 4E). We also found that immunoprecipitated CypD by Parkin was remarkably increased in cardiomyocytes treated with $100\ \mu\text{M}$ H_2O_2 but was not detected in cardiomyocytes treated with $500\ \mu\text{M}$ H_2O_2 (Fig. 4F).

Moreover, the combination of Parkin and CypD was identified in cardiomyocytes exposed to CCCP (Supplementary Fig. S4). Taken together, these results indicate that Parkin could directly bind to but does not affect the expression level of CypD in the mitochondria of cardiomyocytes under oxidative stress.

Parkin catalyzes the ubiquitination of CypD under oxidative stress

Previous studies have revealed that Parkin functions as an E3 ubiquitin-protein ligase involved in the ubiquitin-proteasome-dependent pathway (47). Therefore, we examined whether CypD is ubiquitinated by Parkin. HEK-293 cells were transfected with vectors expressing Myc-CypD together with or without vectors expressing HA-ubiquitin or GFP-Parkin or mito-GFP-Parkin or mito-GFP. Immunoprecipitation and Western blotting revealed that the polyubiquitination levels of CypD increased in the presence of Parkin (Fig. 5A). We then examined whether CypD ubiquitination was enhanced by H_2O_2 . Cardiomyocytes were transfected with vectors expressing HA-ubiquitin and treated with 100 or $500\ \mu\text{M}$ H_2O_2 . Immunoprecipitation analysis indicated that the ubiquitination levels of CypD increased remarkably in response to $100\ \mu\text{M}$ but not $500\ \mu\text{M}$ H_2O_2 treatment (Fig. 5B). The knockdown of Parkin significantly reduced the ubiquitination of CypD (Fig. 5C).

The results just cited revealed that Parkin triggered the polyubiquitination of CypD. K48- and K63-linked polyubiquitination are two common types of polyubiquitination (21). We examined which type of polyubiquitination is triggered by Parkin. HEK-293 cells were transfected with vectors expressing Myc-CypD along with mito-GFP-Parkin and various HA-ubiquitin vectors that express wild-type ubiquitin or ubiquitin mutants containing arginine substitutions of all lysine residues, except those at position 48 (Ub-K48) or 63 (Ub-K63). Immunoprecipitation assays showed that Parkin could polyubiquitinate CypD in the presence of wild-type or K63 ubiquitin but not K48 ubiquitin (Fig. 5D). These results suggest that Parkin induces the polyubiquitination of CypD mainly through K63-linked ubiquitin chains. K48-linked ubiquitin chains can signal the destruction of the protein by proteasome (21). K63-linked linear ubiquitination has been reported to be a degradation-independent function (21, 61). Taken together, our results suggest that the activity of CypD is regulated by K-63-linked polyubiquitination but not its degeneration.

Parkin-mediated inhibition of necrosis and mPTP opening is dependent on CypD

We further explored whether Parkin regulates necrosis and mPTP opening through CypD. The role of CypD in necrotic cell death and mPTP opening in cardiomyocytes was explored. Knockdown of endogenous CypD (Fig. 6A) diminished H_2O_2 -induced mPTP opening and necrosis (Fig. 6B, C) but not apoptosis (Supplementary Fig. S5). We wondered whether Parkin regulated necrosis in cardiomyocytes by targeting CypD. The overexpression of Parkin inhibited the mPTP opening and necrotic cell death induced by $500\ \mu\text{M}$ H_2O_2 ; however, this effect was significantly attenuated by CypD overexpression (Fig. 6D, F). In addition, the knockdown of Parkin strengthened low-dose H_2O_2 -induced mPTP opening and cell necrosis, which were disrupted by the knockdown of CypD (Fig. 6G, H).

CypD is not involved in Parkin-mediated mitophagy under oxidative stress

Parkin participates in oxidative stress-induced mitophagy, which led us to consider whether this protein promotes

FIG. 4. Interaction analysis of Parkin and CypD. (A) Protein expression levels of CypD were detected by immunoblot in cardiomyocytes with H_2O_2 treatment. $n=4$ experiments per group. Quantification is shown in the lower panel. (B) Protein levels of CypD were detected in cardiomyocytes infected with Parkin or β -gal adenovirus. $n=3$ experiments per group. Quantification is shown in the lower panel. * $p < 0.05$. (C) Protein levels of CypD were detected in cardiomyocytes infected with Parkin siRNA or Parkin-sc adenovirus. $n=3$ experiments per group. Quantification is shown in the lower panel. * $p < 0.05$. (D) IP was performed to analyze the interaction of Parkin and CypD. Myc-CypD was immunoprecipitated by GFP-Parkin or mito-GFP-Parkin in HEK293 cells. $n=3$ experiments per group. (E) Enforced expression of Parkin increased the interaction of Parkin with CypD. The interaction of Parkin with CypD was detected by performing IP in cardiomyocytes infected with Parkin overexpression adenovirus or β -gal overexpression adenovirus. $n=3$ experiments per group. (F) The interaction of Parkin with CypD was assessed by IP in cardiomyocytes treated with 100 or 500 μM H_2O_2 for 6 h. $n=3$ experiments per group. β -gal, β -galactosidase; CypD, cyclophilin D; IP, immunoprecipitation.

mitophagy by targeting CypD. We first tested the effect of CypD on mitophagy. GFP-LC3-positive cells induced by low-dose H_2O_2 were not influenced by the inhibition of CypD (Fig. 7A). In addition, low-dose H_2O_2 -induced increase in the levels of LC3-II was not changed on CypD overexpression (Fig. 7B). The inhibition of Parkin attenuated low-dose H_2O_2 -induced mitophagy, which was not varied by the knockdown or overexpression of CypD (Fig. 7C, D). We also found that CCCP-induced mitophagy was not affected by the inhibition of CypD (Supplementary Fig. S6). These results indicate that CypD is not involved in oxidative stress-induced mitophagy in cardiomyocytes and that Parkin can promote mitophagy independent of CypD.

Parkin regulates necrotic cell death in the heart

Having demonstrated that Parkin was involved in H_2O_2 -induced necrosis by targeting CypD *in vitro*, we further investigated its role in the pathogenesis of cardiac infarction in an animal model. We generated Parkin transgenic mice whose hearts showed an increased level of Parkin compared with those of wild-type mice (Supplementary Fig. S7A).

After I/R surgery, the hearts of these transgenic mice exhibited higher levels of Parkin than that in hearts of wild-type mice (Fig. 8A). Promotion of CypD ubiquitination and inhibition of mPTP opening were observed in hearts of Parkin transgenic mice (Fig. 8B, C). In response to I/R injury, Parkin

FIG. 5. Parkin can ubiquitinate CypD on oxidative stress. (A) Parkin can catalyze ubiquitination of CypD. HEK293 cells were transfected with expression vectors encoding Myc-CypD along with HA-ubiquitin, and GFP-Parkin or mito-GFP-Parkin or GFP, respectively. Ubiquitination of CypD was analyzed by IP. $n=3$ experiments per group. Quantification is shown in the lower panel. $*p<0.05$. (B) Ubiquitination of CypD was assessed by IP in cardiomyocytes treated with 100 or 500 μM H_2O_2 for 6 h. $n=3$ experiments per group. Quantification is shown in the lower panel. $*p<0.05$. (C) Knockdown of Parkin inhibited ubiquitination of CypD. Cardiomyocytes were transfected with HA-ubiquitin vector and then infected with Parkin-si or Parkin-sc adenovirus. Ubiquitination of CypD was detected by performing IP in cardiomyocytes treated with 100 μM H_2O_2 . $n=3$ experiments per group. Quantification is shown in the right panel. $*p<0.05$. (D) Parkin can catalyze K63-linked polyubiquitination of CypD. HEK293 cells were transfected with expression vectors encoding Myc-CypD along with mito-GFP-Parkin and HA-ubiquitin or K48 ubiquitin or K63 ubiquitin. Ubiquitination of CypD was analyzed by IP. Results are representative of three independent experiments. $n=3$ experiments per group. Quantification is shown in the lower panel. $*p<0.05$.

transgenic mice exhibited high levels of mitophagy than that in wide-type mice (Supplementary Fig. S7B, C). In addition, the hearts of transgenic mice showed reduced myocardial infarct size (Fig. 8D) and suppressed myocardial necrosis (Fig. 8E) but not altered myocardial apoptosis levels, as analyzed by a terminal dextranucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay (Supplementary Fig. S7D).

Next, cardiac function in animal models were detected. Parkin transgenic mice showed improved cardiac remodeling and function, as assessed by myocardial cross-section area

(Fig. 8F), collagen content (Fig. 8G), and cardiac function (Fig. 8H). Taken together, these results indicate that Parkin participates in the maintenance of cardiac survival and function by inhibiting cardiomyocyte necrosis (Supplementary Fig. S8).

Discussion

Necrosis has been demonstrated to be one of the main forms of cell death in heart diseases (1, 17). This study

FIG. 6. Parkin inhibits necrosis and mPTP opening dependent on CypD. (A) The protein levels of CypD were analyzed by immunoblot in cardiomyocytes transfected with CypD-siRNA (CypD-si) or its scramble form (CypD-sc). $n = 3$ experiments per group. Quantification is shown in the lower panel. $*p < 0.05$. (B, C) Knockdown of CypD prevents H₂O₂-induced mPTP opening and necrosis. Cardiomyocytes were exposed to 500 μ M H₂O₂ for 12 h and then stained by TMRM to analyze mPTP opening (B). Necrotic cell death was detected by using PI staining assay in cardiomyocytes exposed to 500 μ M H₂O₂ for 24 h (C). $*p < 0.05$ versus H₂O₂ alone. $n = 3$ experiments per group. (D) The protein levels of CypD were analyzed in cardiomyocytes infected with CypD or β -gal adenovirus. $n = 4$ experiments per group. Quantification is shown in the lower panel. $*p < 0.05$. (E, F) Enforced expression of Parkin inhibited mPTP opening (E) and necrosis (F) in cardiomyocytes exposed to 500 μ M H₂O₂, which was attenuated by overexpression of CypD. $*p < 0.05$ versus Parkin alone treated with H₂O₂; # $p < 0.05$ versus H₂O₂ alone. $n = 3$ experiments per group. (G, H) Knockdown of CypD can attenuate Parkin-siRNA increased mPTP opening and necrosis. Cardiomyocytes were transfected with Parkin-siRNA, transfected with CypD siRNA, and finally treated with 100 μ M H₂O₂ for 24 h. mPTP opening (G) and necrosis (H) were analyzed as described. $*p < 0.05$ versus Parkin-siRNA alone treated with H₂O₂; # $p < 0.05$ versus H₂O₂ alone. Data are expressed as mean $\pm$ SD, $n = 3$ experiments per group.

reveals the protective effect of Parkin against both oxidative stress- and ischemia-triggered cardiac injury by inhibiting cardiomyocyte necrosis. We found that Parkin negatively regulates necrotic cell death by preventing mPTP opening, which is the main mechanism of necrosis. We also identified CypD as a substrate of Parkin, and CypD was ubiquitinated by Parkin. In addition, Parkin participates in oxidative stress-induced mitophagy in cardiomyocytes independent of CypD. Briefly, our results unveil a novel signaling pathway of cardiac necrosis regulation, and Parkin may be important for blocking the effect of pathological insults on the heart.

Our results showed that Parkin played a cardiac protection role through inhibiting necrosis and promoting mitophagy under moderate oxidative stress. Under severe oxidative stress, Parkin was downregulated, which led to reduced mitophagy, increased necrosis and myocardial damage. Some studies have

shown that a relationship exists between autophagy and necrosis. For example, autophagy regulated gene 16L (Atg16L) was found to suppress necrosis, and RIPK1 could be degraded by the autophagy process. RIPK1-mediated ERK phosphorylation of TFEB prevents autophagy, and RIPK3 inhibits autophagy by interrupting LC3-p62 interaction (39). However, it is unknown whether Parkin-regulated autophagy and Parkin-regulated necrosis are related, which is a meaningful question. We first demonstrated that Parkin regulated autophagy and necrosis in two independent manners.

Parkin encodes a 465-amino-acid protein with a ubiquitin-like domain at the N-terminus and two ring-finger motifs at the C-terminus (23). Parkin functions as an E3 ubiquitin-protein ligase involved in the ubiquitin-proteasome-dependent pathway (47). Previous studies have demonstrated that Parkin is highly expressed in mature heart. Our results show that

FIG. 7. Parkin induces mitophagy independent of CypD. (A) Knockdown of CypD did not affect the number of GFP-LC3 puncta-positive cells. Cardiomyocytes were exposed to 100 μM H₂O₂ for 6 h after being transfected with GFP-LC3 plasmid. GFP-LC3 puncta-positive cells were quantified. *n* = 3 experiments per group. (B) The increasing level of LC3-II induced by 100 μM H₂O₂ was not influenced by overexpression of CypD. *n* = 3 experiments per group. (C, D) Knockdown of Parkin inhibited mitophagy of cardiomyocytes treated with 100 μM H₂O₂, which was not influenced by either knockdown (C) or overexpression of CypD (D). Data are expressed as mean ± SD, *n* = 3 experiments per group.

Parkin was downregulated in cardiomyocytes treated with H₂O₂ and in hearts subjected to I/R. We also found that Parkin participated in the maintenance of cardiac survival and function. Similarly, other studies have obtained the same results. The enforced expression of Parkin in isolated cardiomyocytes prevented hypoxia-induced cell death, whereas Parkin-deficient mice exhibited enhanced sensitivity to MI (20, 24). The cardioprotective effect of Parkin was mainly due to its crucial role in the clearance of damaged mitochondria (24). The dysregulation of Parkin-mediated mitophagy may contribute to heart injury (15, 20). Intriguingly, we first demonstrated that Parkin protects against oxidative stress- and ischemia-triggered cardiac injury by inhibiting cardiomyocyte necrosis.

Parkin was first identified as a PD-associated gene (23). Numerous studies have implicated Parkin in neurodegenerative diseases, including PD (23) and Alzheimer's disease (AD) (33). Parkin also plays a role in cancer development (3). Whether Parkin regulates necrosis in other systems or pathological processes, such as neurodegenerative diseases and cancer, is an interesting question for future exploration. Loss-of-function mutations in the *PARK2* gene are related to early-onset familial PD (23). Notably, *PARK2* gene alterations are not restricted to PD but also occur frequently in a wide variety of malignancies (51). Additional studies are needed to determine whether mutations in the *PARK2* gene are related to cardiovascular disease in the human population and whether PD patients are susceptible to heart disease.

Emerging evidence has revealed several mechanisms by which necrotic cell death is regulated, including RIP1/RIP3-dependent (29) and mPTP-CypD-dependent (27) death sig-

naling pathways. Nevertheless, the fundamental mechanisms underlying myocardial necroptosis, especially the mPTP-CypD-dependent death pathway, are poorly understood. In this study, we found that Parkin inhibited oxidative stress-induced mPTP opening in a CypD-dependent manner and could catalyze the ubiquitination of CypD under oxidative stress. It has been reported that CypD-deficient (knockout) mice showed a reduction in the number of necrotic cells and a high level of resistance to myocardial damage, including reduced infarct area and improved cardiac function, on I/R injury (27, 38, 55), which is similar to that of Parkin-transgenic mice.

The molecular structure of mPTP remains unclear, and different models have been proposed. A classic model of mPTP consists of a dimer of voltage-dependent anion channels (VDAC) in the OMM and a dimer of adenine nucleotide translocase (ANT) in the mitochondrial IMM (36). In contrast, CypD in the mitochondrial MA is the essential regulator of mPTP pore opening and the only genetically confirmed indispensable mPTP component (46). The binding of CypD to ANT initiates a tunnel-like structure that connects the mitochondrial MA with the cytosol (28). The therapeutic value of maintaining mPTP in a closed state is highlighted by the resistance of *Ppif* (the gene encoding CypD) knockout mice (*Ppif*^{-/-}) to a variety of diseases, such as muscular dystrophy (35) and AD (7), as well as acute cellular loss after MI and stroke (9, 50). Thus, understanding how mPTP is regulated could help to identify ways to specifically inhibit mPTP opening.

In this work, we identified Parkin as a negative regulator of CypD by directly ubiquitinating CypD. In a recent study, Moll and colleagues (50) discovered that oxidative stress triggers the binding of p53 to CypD and that this interaction promotes CypD-dependent mPTP opening and necrosis, which was implicated in brain I/R injury. Other proteins, such as STAT3 and amyloid β protein, have also been reported to participate in mPTP activation by interacting with CypD (8, 34). In addition to the modulation of CypD by various proteins, post-translational modifications also contribute to its regulation. Deacetylation by Sirt3 (18) and S-nitrosylation (41) modulated the activity of CypD and mPTP induction. Phosphorylation of CypD by PGAM5 contributed to mPTP opening (63). These studies suggest that CypD is likely regulated in a cell type- and stimulation-dependent manner during necrosis. We first identified the ubiquitination of CypD in cardiomyocytes. Whether the regulation of CypD ubiquitination is involved in other cell types and stimuli needs to be further investigated.

Previous studies have yielded conflicting conclusions on Parkin subcellular localization, showing its localization in the cytosol, the trans-Golgi network, the synaptic membrane compartment, postsynaptic densities, lipid rafts, the endoplasmic reticulum, the nuclear MA, and mitochondria (6, 10, 48). In this study, we examined the subcellular localization of endogenous Parkin in cardiomyocytes. Consistent with most studies, we found that endogenous Parkin was predominantly located in the cytosol under normal conditions and recruited to damaged mitochondria under oxidative stress. To date, the majority of research has focused on the function of the mitophagy of Parkin, which presents a limitation.

When mitochondria are damaged, PINK1 accumulates and recruits Parkin to the OMM to induce mitophagy (52). A number of OM substrates, such as VDAC1 (12), Mfn1, and Mfn2, are ubiquitinated by Parkin (11). This modification

FIG. 8. Parkin regulates necrotic cell death in heart. (A) Parkin protein levels in Parkin transgenic (Tg) and wild-type (WT) mice exposed to sham operation or I/R. $n = 3$ mice per group. Quantification is shown in the lower panel. * $p < 0.05$. (B) The ubiquitination of CypD was measured by IP in Parkin Tg and WT mice exposed to sham operation or I/R injury. $n = 5$ mice per group. (C) Calcium-induced mitochondrial swelling was delayed by overexpression of Parkin. Total cardiac mitochondria isolated from wild-type (WT) and Parkin transgenic mice (Tg) were exposed to 200 μ M Ca^{2+} to induce swelling or left untreated. The change in absorbance that occurs as a result of mitochondrial swelling is monitored over time at 520 nm and is expressed relative to the absorbance at the beginning of the assay ($n = 3$ preparations per group). (D) Overexpression of Parkin attenuated myocardial infarct size on I/R. Parkin Tg or WT mice were subjected to 45 min of LAD coronary artery ligation followed by 1 h of reperfusion. The areas of myocardial INF, AAR, and LV myocardial infarct sizes were measured and assessed 24 h after reperfusion. The ratios of AAR to LV and INF to AAR are shown ($n = 8$ mice per group). * $p < 0.05$ versus WT + I/R. (E) Myocardial necrosis was detected in I/R heart. PI was injected into the mice to label necrotic cells 1 h before I/R. Mice were subjected to LAD ligation for 45 min and then reperfusion for 1 h. Representative images of ventricular myocardium sections are shown on the left, and quantitative analysis of PI-positive cells is shown on the right. Red, PI-positive myocyte nuclei; blue, DAPI-stained nuclei ($n = 6$ mice per group). * $p < 0.05$ versus WT + I/R. (F, G) Analysis of cross-sectional area and collagen areas of WT and Parkin Tg mice subjected to I/R was done. Representative photomicrographs show tetramethyl rhodamine isothiocyanate-conjugated wheat germ agglutinin staining (left). The cross-sectional area was quantified (right) (D). Collagen deposition was detected by Masson's trichrome staining (left). Collagen area was quantitatively analyzed (right) (E). Data are mean $\pm$ SD ($n = 8$ mice per group). * $p < 0.05$. (H) Echocardiographic analysis of left ventricular dimensions and cardiac function in mice exposed to I/R. LVIDs, systolic left ventricular internal diameter; LVIDd, diastolic left ventricular internal diameter; FS, fractional shortening of left ventricular diameter, calculated as $[(LVIDd - LVIDs)/LVIDd] \times 100$. $N = 8$ mice per group, * $p < 0.05$. AAR, area at risk; I/R, ischemia/reperfusion; INF, infarction; LAD, left anterior descending; LV, left ventricle; Tg, transgenic; WT, wild-type. Color images are available online.

recruits the autophagy adaptor molecule p62/SQSTM1 and thereby targets mitochondria for autophagic removal (12). In fact, the intramitochondrial localization of endogenous Parkin was also confirmed by some studies (25, 45). We also demonstrated that Parkin could localize to the MA of mitochondria under oxidative stress and CCCP treatment. However, the function of Parkin in the mitochondrial MA is unclear. CypD is a mitochondrial MA protein (50). Our present study shows that Parkin is a CypD-interacting partner under oxidative stress conditions in cardiomyocytes. We also demonstrated that Parkin is involved in mitophagy in cardiomyocytes under the same conditions. Thus, a fraction of Parkin accumulated in the mitochondrial MA, whereas another portion also localized to the surface (OM) in response to oxidative stress.

However, our results show that CypD was not involved in oxidative stress-induced mitophagy and that Parkin could promote mitophagy independent of CypD. We speculate that the mechanism by which Parkin protects against oxidative stress-induced cardiac injury is by promoting autophagy and preventing necrosis, independently. The function and molecular mechanism of Parkin may be different at different intracellular localizations. Indeed, several recent studies have shown that Parkin participates in a wide array of cellular processes, such as the regulation of lipid metabolism (4), cytokine production during infection, and pathogen defense (32). Our results provide a new clue for the understanding of the Parkin-controlled cellular events beyond mitochondrial homeostasis. The function and regulation of Parkin in the mitochondrial MA need to be further investigated.

This work highlights the unexpected connection between mitochondrial MA-localized Parkin and the mPTP-CypD-dependent necrotic signaling pathway in cardiac injury. Parkin acts as a negative regulator of CypD by directly ubiquitinating CypD. Our results revealed a novel myocardial necrotic regulating model composed of Parkin, CypD, and mPTP, which suggests that the modulation of Parkin may provide potential therapeutic targets and strategies to treat myocardial necrosis-referred heart diseases, including MI and HF. Further studies are needed to clarify the upstream regulator of Parkin and to determine how this pathway is initiated on stress.

Materials and Methods

Cell culture and treatment

Neonatal rat cardiomyocytes were isolated from 1- to 2-day-old Sprague Dawley rats. In brief, after dissection, hearts were washed and then minced in phosphate buffered saline (PBS) solution (pH adjusted to 7.2). We dispersed the tissues in a series of incubations at 37°C in PBS solution containing 1.2 mg/mL pancreatin and 0.14 mg/mL collagenase type II (Worthington, Lakewood, NJ). After termination of digestion in fetal bovine serum, the cells were collected by centrifugation at 1000 rpm for 5 min. Then, cells were preplated in Dulbecco's modified Eagle medium/F-12 (Thermofisher Scientific, Gibco, MA) supplemented with 5% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C for 1 h to remove the noncardiomyocytes. The suspended cells were collected and plated at 0.01 mg/mL in laminin-coated culture dishes in Dulbecco's modified Eagle medium/F-12 (Thermofisher Scientific, Gibco, MA) supplemented with 5% heat-inactivated fetal bovine serum, 100 U/mL

penicillin, 100 µg/mL streptomycin, and 0.1 mM bromodeoxyuridine.

Cardiomyocytes were treated with 100 or 500 µM H₂O₂, except as otherwise indicated elsewhere. Cardiomyocytes were treated with 50 µM CCCP. For TNF-α-stimulated cells, cardiomyocytes were exposed to 10 ng/mL human TNF-α (Sigma-Aldrich, St. Louis, MO). To inhibit apoptosis, cells were treated with 20 µM apoptosis inhibitor Z-VAD-fmk (Promega, Madison, WI). HEK293 cells were cultured in Dulbecco's modified Eagle medium (Thermofisher Scientific, Gibco, MA) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 110 mg/mL sodium pyruvate in a humidified atmosphere containing 5% CO₂ at 37°C.

Generation of Parkin transgenic mice

Parkin transgenic mice were generated as previously described (53). Murine Parkin cDNAs were obtained from mouse heart through real time-polymerase chain reaction (RT-PCR) and cloned into pzMHC-clone26 vector under the control of the α-myosin heavy chain promoter. After linearization and purification, the recombinant vectors were microinjected into 0.5 days post coitum zygote. The genotyping of Parkin transgenic mice was identified by PCR.

Cell death assay

The cell death assay was performed as previously described (54). Propidium iodide (PI) was used to assess necrosis. Apoptotic cell death was assessed by TUNEL assay by using an *in situ* cell death detection kit (Roche, Hamburg, Germany). The detection procedure was performed in accordance with the manufacturer's instructions. Necrosis was also measured by assessing supernatant lactate dehydrogenase (LDH) activity. A spectrophotometric kit (Nanjing Jiancheng, Jiangsu, China) was used according to the manufacturer's instructions. In brief, 20 µL supernatant was collected in a 96-well plate, then successively added to 25 µL MA buffer and 5 µL coenzyme I, and incubated at 37°C for 15 min. Then, 25 µL 2,4-dinitrophenylhydrazine was added, and the mixture was incubated at 37°C for 15 min. Then, 250 µL 0.4 M NaOH was added and the reaction was incubated at room temperature for 5 min. The absorbance value was measured at 450 nm with a spectrophotometer (BioTek, VT), and the LDH activity was calculated. Caspase-3 activity was measured by using an Apo-ONE homogeneous caspase-3/7 assay kit (Promega, Madison, WI) according to the manufacturer's protocol.

Mitochondrial staining and immunofluorescence

Cells were plated onto coverslips precoated with 0.01% poly-L-lysine. Then, they were treated with 0.02 µM Mito-Tracker Red CMXRos (Molecular Probes, Eugene, OR) at 37°C for 20 min and subsequently fixed and processed for immunofluorescence. The cells were fixed with cold methanol for 5 min at -20°C. After being washed with PBS, cells were blocked with 5% bovine serum albumin for 1 h, followed by incubation with primary antibody against Parkin (ab15954; Abcam, Cambridge, United Kingdom) overnight at 4°C. Cells were then washed with PBS and incubated in a dark chamber with the fluorescein isothiocyanate-conjugated secondary antibody (Sigma-Aldrich, St. Louis, MO) at room

temperature for 2 h. Subsequently, the slides were incubated with the nucleic acid-specific stain 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). The samples were imaged by using a laser scanning confocal microscope (Zeiss LSM 510 Meta).

GFP-LC3 puncta detection

Autophagy was evidenced by the formation of LC3-positive puncta structures, which are essential for the dynamic process of autophagosome formation. Cells were transfected with GFP-tagged LC3 plasmid by using Lipofectamine reagent (ThermoFisher Scientific, Invitrogen, MA) according to the manufacturer's instructions. GFP-LC3 fluorescence was imaged by using a laser scanning confocal microscope (Zeiss LSM 510 Meta). The number of GFP-LC3 puncta cells was quantified.

Adenoviral constructs

Parkin and CypD adenoviral constructs were prepared as previously described (54). In brief, rat Parkin and CypD cDNAs were reverse transcribed from total RNA of the rat heart and then cloned into the Adeno-X™ Expression System (Clontech, Mountain View, CA) according to the manufacturer's instructions. Adenoviruses were produced and amplified in HEK293 cells. The adenovirus containing β -galactosidase was prepared as a control. All constructs were amplified in HEK293 cells. Cardiomyocytes were infected with adenoviruses at a multiplicity of infection (moi) of 150 in the absence of fetal bovine serum for 2 h.

siRNA construction of parkin and CypD

Parkin siRNA sense sequence is 5'-TTCCAAACCGGA TGAGTGG-3'; the antisense sequence is 5'-CCACTCAT CCGTTTGGAA-3'. The scramble *parkin* siRNA sense sequence is 5'-GCTCATCGAGCTAGTAGAG-3'; the scramble antisense sequence is 5'-CTCTACTAGCTCGATGAGC-3'. The CypD siRNA sense sequence is 5'-CACCAATGGCT CTCAGTTC-3'; the antisense sequence is 5'-GAACTGA GAGCCATTGGTG-3'. The scramble CypD siRNA sense sequence is 5'-CGTCACTGTCACTACATGC-3'; the scramble antisense sequence is 5'-GCATGTAGTGACAGTGACG-3'. These siRNAs were cloned into the pSilencer 1.0-CMV System (Ambion, Grand Island, NY) according to the manufacturer's instructions. Adenoviruses were produced and amplified in HEK293 cells.

Mitochondrial membrane potential ($\Delta\psi$ m) detection

Cells were incubated with 100 nM tetramethylrhodamine methylester (TMRM) (Molecular Probes, ThermoFisher Scientific, Invitrogen) at 37°C for 15 min. The red TMRM fluorescence was detected by confocal microscopy, and the fluorescence intensity was measured by the 543/590 excitation/emission filter set.

Quantitative RT-PCR

Total RNA was extracted by Trizol reagent (ThermoFisher Scientific, Invitrogen). The RNAs were reverse transcribed into cDNA with oligo-d(T) or random primers by using a reverse transcriptase kit (Takara, Otsu, Japan). The expres-

sion levels of Parkin mRNA were detected by quantitative RT-PCR (qRT-PCR) using a SYBR® Premix Ex Taq™ (Takara, Otsu, Japan) on a CFX96™ Real-Time System instrument (Bio-Rad, Hercules, CA). The qRT-PCR results were normalized to those of GAPDH. The primers used for rat GAPDH were forward: 5'-TGGAGTCTACTGGCGT CTT-3'; reverse: 5'-TGTCATATTTCTCGTGGTTCA-3'. The primers used for mouse GAPDH were forward: 5'-TGTGTCCGTCGTGGATCTGA-3'; reverse: 5'-CCTGCTT CACCACCTTCTTGA-3'. The primers used for rat Parkin were forward: 5'-CTTGACTCGAGTGGACCTCA-3'; reverse: 5'-GGTTTAGCTGCTGGACCTCT-3'. The primers used for mouse Parkin were forward: 5'-AGAGGAAAGT CACCTGCGAA-3'; reverse: 5'-TGTAGGCCCTGAGAAAGT GGCT-3'. The primers used for rat CypD were forward: 5'-AGACAGACTGGCTGGATGG-3'; reverse: 5'-TAGCTCA ACTGGCCACAGTC-3'.

Immunoblotting

Immunoblotting was carried out as previously described (49). In brief, the cells were collected and lysed on ice for 1 h by using RIPA lysis buffer (Solarbio, Beijing, China) containing 0.1 mM phenylmethanesulfonyl fluoride (PMSF) and a protease inhibitor (Roche). The total protein was prepared after centrifugation. The samples were subjected to 10–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the molecular weight of protein. After being transferred to nitrocellulose membranes, the blots were probed with primary antibodies, including anti-Parkin antibody (Abcam; ab15954), anti-LC3A/B antibody (Cell Signaling Technology, Boston, MA; #4108), and anti-CypD (Abcam; ab110324). The antigen-antibody complexes were visualized by the Pierce® ECL Western Blotting Substrate (Pierce, Rockford, IL) according to the manufacturer's instructions and exposed on X-ray film (Kodak, Rochester, NY). Quantification of immunoblotting data was analyzed by Image J software.

Immunoprecipitation

Immunoprecipitation was carried out as previously described (54). The cells were incubated with 500 μ L NP-40 lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1% NP-40, 10% glycerol, 0.2 mM PMSF, and protease inhibitor) at 4°C for 30 min. After centrifugation, 50- μ L samples were aliquoted for input, and the remaining samples were used for immunoprecipitation. The samples were incubated with 2 μ g anti-GFP antibody and 50 μ L Protein-A/G PLUS-Agarose (Santa Cruz Biotechnology, CA), rotating at 4°C overnight. Then, the beads were successively treated with the following steps: washing thrice with 1 mL low-salt NP40 lysis buffer (300 mM NaCl), washing twice with 1 mL high-salt lysis buffer (500 mM NaCl), mixing with 25 μ L 2 $\times$ sample buffer, and boiling for 10 min. The released proteins were subjected to 12% SDS-PAGE and detected by immunoblotting using an anti-CypD antibody as described earlier.

Preparation of submitochondrial fractions

Submitochondrial fractions were prepared as described elsewhere (50). First, mitochondria were isolated from

cultured cells by using a sucrose gradient separation assay. Then, submitochondrial fractions were prepared by phosphate swelling-shrinking. Briefly, purified mitochondrial pellets were resuspended in swelling buffer (10 mM KH_2PO_4 , pH 7.4, and protease inhibitor) and incubated at 4°C for 15 min with gentle mixing. Then, the resuspension was mixed with an equal volume of shrinking buffer (10 mM KH_2PO_4 , pH 7.4, 32% sucrose, 30% glycerol, 10 mM MgCl_2 , and protease inhibitor) at 4°C for 15 min. The supernatant containing OM and intermembrane space (IMS) fractions was collected after centrifugation at 10,000 g for 10 min. After washing three times with a 1:1 mixture of swelling-shrinking buffer, the pellets containing IM and MA were resuspended in swelling buffer and sonicated to disrupt the IM. OM + IMS and IM + MA were further fractionated by centrifugation at 120,000 g at 4°C for 1 h. The pellets were collected as OM and IM fractions, respectively. The supernatants were collected as IMS fractions and MA fractions after being concentrated by using Centricon 10K and Microcon 10K centrifugal filters (Merk Millipore, Darmstadt, Germany), respectively.

Transmission electron microscopy

Conventional electron microscopy was performed as previously described (54). Briefly, the cells were successively treated with 2.5% glutaraldehyde, 1% osmium tetroxide, and a graded series of ethanol concentrations. Then, the samples were embedded in Embed812 resin and subjected to ultramicrotomy. After being mounted on copper grids, the samples were stained with uranyl acetate and lead citrate and photographed with an FEI Tecnai spirit transmission electron microscope. Heart ultrastructural analysis was also performed. The number of autophagic vacuoles was determined for a minimum of 100 cells. The investigator who assessed the autophagic vesicle was blinded to the sample identity.

Mouse model of myocardial I/R and determination of infarct sizes

An I/R experiment and determination of infarct sizes were carried out as previously described (53, 54). All animal experiments were performed by using protocols that adhered to institutional guidelines and were approved by the Institute Animal Care Committee. Adult C57BL/6 mice (Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China) at 10–12 weeks of age were randomly subjected to I/R surgery. In brief, the mice were anesthetized with an intraperitoneal injection of 400 mg/kg chloral hydrate and subjected to thoracotomy with a trachea cannula to provide artificial ventilation (0.2 mL tidal volume, 110 breaths/min) with a rodent ventilator supplemented with 100% oxygen. After the pericardial sac was removed, the left anterior descending (LAD) coronary artery was exposed and occluded by an 8-0 silk suture passed around it at the inferior border of the left auricle. The mice were subjected to 45 min of LAD ligation, followed by 1 h of reperfusion. The same procedure was performed on sham-operated mice without ligating the suture.

At 24 h after reperfusion, infarct sizes were determined. The mice were anesthetized and again subjected to thoracotomy. The LAD was reoccluded at the same original site. Evans Blue (1 mL of a 2.0% solution; Sigma-Aldrich) was injected through the jugular vein into the heart to delineate

the ischemic zone from the nonischemic zone. Then, the hearts were rapidly excised and sectioned at 1 mm thickness. After incubation in 1.0% 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich) for 15 min at 37°C, the infarcted myocardium was stained white, the risk zone was stained red, and the noninfarct tissue remained blue. Ice-cold sterile saline was used to stop the staining, and then the slices were fixed in 10% neutral buffered formaldehyde. Each slice was individually photographed on both sides. The areas of nonischemic left ventricle (LV), area at risk (AAR), and infarction (INF) were analyzed by using computer-assisted planimetry (ImageJ) by an observer blinded to sample identity. The ratios of AAR/LV, INF/AAR, and INF/LV were calculated.

PI and TUNEL staining of heart tissue

The detection of necrotic cells was carried out as previously described (54). Briefly, mice were injected with PI (10 mg/kg) 1 h before I/R termination to label the necrotic cells. Then, the hearts underwent the following steps: explantation, snap-freezing in liquid nitrogen, and sectioning at 6 μm thickness after I/R termination. TUNEL staining was performed by using an *in situ* cell death detection kit (Roche) according to the manufacturer's instructions; α -actinin was used to identify cardiomyocytes, and DAPI was used to stain nuclei. We photographed the slices at 400 $\times$ magnification by using a laser scanning confocal microscope (Zeiss LSM 510 Meta). Then, 100–200 cardiomyocytes in 30–50 fields were chosen and quantified by observers blinded to the sample identification.

Echocardiographic assessment, Masson trichrome staining and tetramethyl rhodamine isothiocyanate-conjugated wheat germ agglutinin staining, and Ca^{2+} -induced mPTP opening assay

Echocardiographic assessment was carried out as previously described (56). In brief, 1 week after I/R surgery, transthoracic echocardiography was performed by using a Vevo 770 high-resolution system (Visualsonics) equipped with a 40 MHz RMV 704 scanhead. Systolic left ventricular internal diameter (LVIDs) and diastolic left ventricular internal diameter (LVIDd) were measured. Fractional shortening of the left ventricular diameter was calculated as $[(\text{LVIDd} - \text{LVIDs})/\text{LVIDd}] \times 100$.

After *in vivo* evaluation of cardiac function, the mice were euthanized, and the hearts were harvested and subjected to Masson trichrome staining, tetramethyl rhodamine isothiocyanate (TRITC)-conjugated wheat germ agglutinin (WGA) staining, and Ca^{2+} -induced mPTP opening assay. Standard Masson trichrome staining (Sigma-Aldrich) was used to assess myocardial fibrosis induced by I/R injury according to the manufacturer's instructions. TRITC-WGA (Sigma-Aldrich) staining was performed to determine the cross-sectional area according to the manufacturer's instructions. mPTP opening in cardiomyocytes from the mice heart tissue was measured in the following manner. Mitochondria were isolated by using a mitochondria extraction kit (Solarbio; SM0020). The isolated mitochondria were resuspended in an assay medium containing 100 mM KCl, 50 mM 3-(N-Morpholino) propanesulfonic acid, 5 mM KH_2PO_4 , 0.5 μM ethylene glycol-bis(β -aminoethylether)-N,N,N',N'-tetraacetic acid, 1 mM MgCl_2 , 5 mM glutamate, and 5 mM malate. Then, the

resuspended mitochondria in swelling buffer (2 mg/mL protein) were warmed to room temperature in a 96-well plate and CaCl_2 (200 μM) was added. The time-dependent decrease in absorbance at 520 nm is indicative of the tendency for mPTP opening and mitochondrial swelling as previously reported (43).

Statistical analysis

All statistical analyses were performed by the SPSS 16.0 statistical software package. Data are expressed as the mean $\pm$ standard deviation of at least three independent experiments. A one-way analysis of variance was used for multiple comparisons. A value of $p < 0.05$ was considered significant.

Author Disclosure Statement

No competing financial interests exist.

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Supplementary Material

Supplementary Figure S1
Supplementary Figure S2
Supplementary Figure S3
Supplementary Figure S4
Supplementary Figure S5
Supplementary Figure S6
Supplementary Figure S7
Supplementary Figure S8

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Abbreviations Used

β -gal = β -galactosidase
AAR = area at risk
AD = Alzheimer's disease
ANT = adenine nucleotide translocase
CCCP = carbonyl cyanide 3-chlorophenylhydrazone
CypD = cyclophilin-D
DAPI = 4',6-diamidino-2-phenylindole
GFP = green fluorescent protein
HF = heart failure
HM = heavy membrane
I/R = ischemia/reperfusion
IM = inner membrane
IMM = inner mitochondrial membrane
IMS = intermembrane space
INF = infarction
LAD = left anterior descending
LC3 = light chain 3
LDH = lactate dehydrogenase
LV = left ventricle
LVIDd = diastolic left ventricular internal diameter
LVIDs = systolic left ventricular internal diameter
MA = matrix
MI = myocardial infarction
mPTP = mitochondrial permeability transition pore
OM = outer membrane
OMM = outer mitochondrial membrane
PBS = phosphate buffered saline
PD = Parkinson's disease
PI = propidium iodide
PMSF = phenylmethanesulfonyl fluoride
qRT-PCR = quantitative real-time polymerase chain reaction
RIPK = receptor-interacting serine/threonine-protein kinase
SD = standard deviation
SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TMRM = tetramethylrhodamine methylester
TNF- α = tumor necrosis factor- α
TRITC = tetramethyl rhodamine isothiocyanate
TUNEL = terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling
VDAC = voltage-dependent anion channels
WGA = wheat germ agglutinin