

Original Paper

Parkin Modulates $ERR\alpha$ /eNOS Signaling Pathway in Endothelial Cells

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Key Words

Parkin • $ERR\alpha$ • eNOS • Ubiquitination • Mitochondrial dysfunction

Abstract

Background/Aims: Although a number of reports documented the important role of parkin in mitophagy, emerging evidence also indicated additional functions of parkin besides mitophagy. The present study was undertaken to investigate the role of parkin in the regulation of $ERR\alpha$ /eNOS pathway in endothelial cells (ECs). **Methods:** Mouse aortic endothelial cells (MAECs) and cardiac muscle HL-1 cells were transfected with parkin plasmid or siRNA. $ERR\alpha$ inhibitor XCT-790, autophagy inhibitor 3-MA and Bafilomycin A1, and caspase inhibitor Z-VAD-FMK were used to block autophagy or apoptosis. Western blotting was performed to examine the protein levels. Flow cytometry was applied to determine the cell apoptosis and ROS production. Mitochondrial membrane potential was measured using JC-1 and TMRM. Immunoprecipitation was performed to confirm the parkin effect on $ERR\alpha$ ubiquitination. **Results:** Overexpression of parkin resulted in a significant reduction of total-eNOS and p-eNOS in parallel with the downregulation of $ERR\alpha$ (a regulator of eNOS) protein and the enhancement of $ERR\alpha$ ubiquitination. To test the role of $ERR\alpha$ in regulating eNOS in this experimental setting, we treated ECs with $ERR\alpha$ inhibitor and found a decrement of total-eNOS and p-eNOS. On the contrary, overexpression of $ERR\alpha$ increased the levels of total-eNOS and p-eNOS. Meanwhile, parkin overexpression induced mitochondrial dysfunction and cell apoptosis in both ECs and HL-1 cells. Finally, we confirmed that the parkin effect on the regulation of eNOS was independent of the autophagy and apoptosis. **Conclusion:** These findings suggested that parkin overexpression downregulated eNOS possibly through the ubiquitination of $ERR\alpha$ in endothelial cells.

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Introduction

It is well known that endothelial cells play important roles in vascular physiology and pathology. Endothelial cells form the barrier with selective permeability, particularly in the brain. Endothelial dysfunction and injury contribute to the pathogenesis of hypertension, atherosclerosis, myocardial infarction, and thromboembolism [1-3]. In vascular endothelial cells, nitric oxide (NO) is an endothelium-derived vasodilator contributing to the blood pressure control and vessel protection. Endothelial NO synthase (eNOS) is constitutively presented in endothelial cells and catalyzes L-arginine to form NO [4]. Therefore, eNOS is of importance in maintaining the normal vascular function.

As an E3-ubiquitin ligase, parkin is important in initiating mitophagy and regulating mitochondrial homeostasis. Mutation of parkin was associated with Parkinson's disease [5]. PINK1 drives parkin to locate in mitochondria and interacts with each other to promote mitophagy. Mitochondrial dysfunction can trigger PINK1 to accumulate and phosphorylate Mfn2 which attracts parkin to ubiquitinate mitochondrial outer membrane proteins for autophagosomal engulfment [6, 7]. Recent studies have revealed that autophagy could protect heart and vasculature against various insults [8-10]. Moreover, evidence also showed that parkin could modulate cell cycle, downregulate VEGFR2, and affect tumorigenic process independently of the mitophagy function [11-13], indicating the multifunction of parkin in the physiology and pathology.

Estrogen-related receptor α (ERR α) is a nuclear orphan receptor and serves as a transcription factor [14]. Peroxisome proliferator activated receptor-gamma coactivator 1 α (PGC-1 α) regulates the expression and activity of ERR α [15]. PGC-1 α and ERR α work with each other to modulate the transcription of target genes involved in mitochondrial metabolism and mitochondrial biogenesis [16, 17]. Recently, a report suggested that eNOS was a target gene of ERR α [18]. More interestingly, another group demonstrated that parkin could degrade ERR α by a ubiquitination mechanism in SH-SY5Y cells [19]. However, the role of parkin in modulating ERR α /eNOS axis in vascular endothelial cells is unknown.

Materials and Methods

Materials

Plasmid of pRK5-Myc-parkin was bought from Addgene. ERR α agonist XCT-790 was from MedChem Express (HY-10426), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, and trypsin solution with EDTA were from Gibco (Invitrogen, Grand island, NY). Lipofectamine™2000 was purchased from Invitrogen (Carlsbad, CA). ROS detection kit (catalog no. S0033), caspase inhibitor Z-VAD-FMK (catalog no. C1202), and JC-1 detection kit (catalog no. C2006) were from Beyotime (Shanghai, China). TMRM kit (catalog no. T668) was from ThermoFisher. Apoptosis detection kit (catalog no. 556547) was from BD Bioscience. LC3 (catalog no. L7543) antibody, flag antibody (catalog no. F1804), HA antibody (catalog no. H6908), 3-Methyladenine A (catalog no. M9281), and carbonyl cyanide 3-chlorophenylhydrazone (catalog no. C2759) were from Sigma (St. Louis, MO). Bafilomycin A1 (catalog no. S1413) was purchased from Selleck. Parkin (catalog no. ab15954) and P62 (catalog no. ab109012) antibodies were provided by Abcam (Cambridge, MA). Antibodies against ERR α (catalog no. 13826s), GAPDH (catalog no. ab9485), and cleaved-caspase-3 (catalog no. 9664s) were purchased from Cell Signaling Technology (Danvers, MA). Antibodies against eNOS (C-20) (catalogno.sc-654) and p-eNOS (ser1177) (catalog no.sc-12972) were purchased from Santa Cruz (Dallas, ST). TOM20 antibody (catalogno.11802-1-AP) was from Proteintech.

Cell culture

Mouse aortic endothelial cells (MAECs) and cardiac muscle HL-I cells were purchased from Jennio Biotech Co. Ltd (Guangzhou, China). Cells were cultured in DMEM with 10% FBS, penicillin (100U/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$) at 37°C in a 5% CO_2 atmosphere, and were cultivated to 60%-70% confluence. Then cells were treated with XCT-790 (10 μM , 24 h) to inhibit $\text{ERR}\alpha$. In other experiments, cells were pretreated with 3-MA, bafilomycin A1 or Z-VAD-FMK for 2 hours before parkin plasmid transfection (24 h) using lipo2000. To silence parkin, cells were transfected with parkin siRNA for 48 hours.

Immunoprecipitation

After transfection, MAECs were washed three times with cold PBS and lysed in RIPA buffer containing the Protease Inhibitor Cocktail and protease inhibitor PMSF for 30 min. Lysates were centrifuged at 12000rpm for 5 min at 4°C, the supernatants with equal amount of total protein were immunoprecipitated with indicated antibodies at 4°C overnight. The protein samples were boiled in SDS-loading buffer for 10 min and were separated by 10% SDS-PAGE. Immunoblotting was performed with primary antibodies against parkin (1:500), $\text{ERR}\alpha$ (1:1000), HA (1:1000), and Flag (1:1000) followed by the incubation with HRP-labeled secondary antibody. Protein bands were visualized using ECL Plus detection reagents with Amersham ECL Detection Systems (Amersham, Buckinghamshire, UK). Densitometric analysis was performed by using Image Lab Software (Bio-Rad, USA). We used 1mg protein and 2 μg antibody for immunoprecipitation.

Western blotting

After treatments, MAEC and HL-1 cells were washed three times with cold PBS and lysed in RIPA buffer (Beyotime, Shanghai, China) containing the Protease Inhibitor Cocktail and protease inhibitor PMSF for 30 min. Lysates were centrifuged at 12000rpm for 5 min at 4°C. The protein samples were boiled in SDS-loading buffer for 10 min and were separated by 10% SDS-PAGE. Immunoblotting was performed with primary antibodies against parkin (1:500), LC3 (1:1000), P62 (1:1000), $\text{ERR}\alpha$ (1:1000), eNOS (1:200), p-eNOS (1:200), TOM20 (1:1000), cleaved-caspase-3 (1:1000), and GAPDH (1:1000) followed by the incubation with HRP-labeled secondary antibody. Protein bands were visualized using ECL Plus detection reagents with Amersham ECL Detection Systems (Amersham, Buckinghamshire, UK). Densitometric analysis was performed using Image Lab Software (Bio-Rad, USA).

Analyses of ROS production and mitochondrial membrane potential (MMP)

To examine the ROS production in MAECs transfected with parkin, dichlorofluorescein diacetate (DCFDA) was used to measure intracellular ROS levels. DCF fluorescence was analyzed by flow cytometry. To determine the mitochondrial MMP in MAECs, JC-1, a molecular probe and tetramethylrhodamine, methyl ester (TMRM) were used according to the manufacturer's instructions. JC-1 and TMRM fluorescence levels were analyzed by flow cytometry. Alteration of MMP is indicated by a decrease of the ratio of red/green fluorescence intensity.

Annexin V/PI double staining for the measurement of cell apoptosis

Cells were pretreated with 3-MA or Z-VAD-FMK for 2 hours before transfection with parkin plasmids or vectors for additional 24 hours. Apoptotic cells were analyzed by the Annexin V-FITC Apoptosis Detection kit (BD) according to the manufacturer's instructions.

Statistical Analysis

All data were expressed as the means \pm SEM and were analyzed by ANOVA followed by Bonferroni's comparison or unpaired Student's t test. $P < 0.05$ was considered significant.

Results

Role of parkin in regulating eNOS in mouse aortic endothelial cells

Nitric oxide (NO) is a critical small molecule in regulating vascular tone, vascular growth, and vascular inflammation. Endothelial nitric oxide synthases (eNOS) is the key enzyme for NO production in endothelium. To define the role of parkin in regulating total-eNOS and

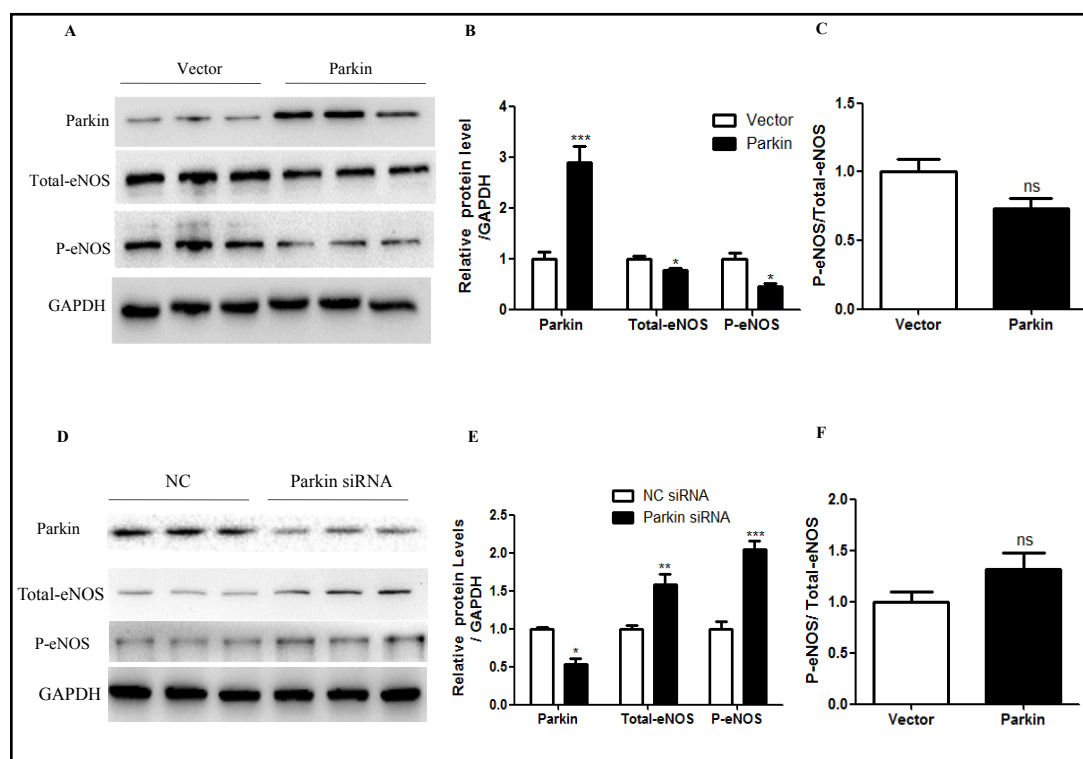


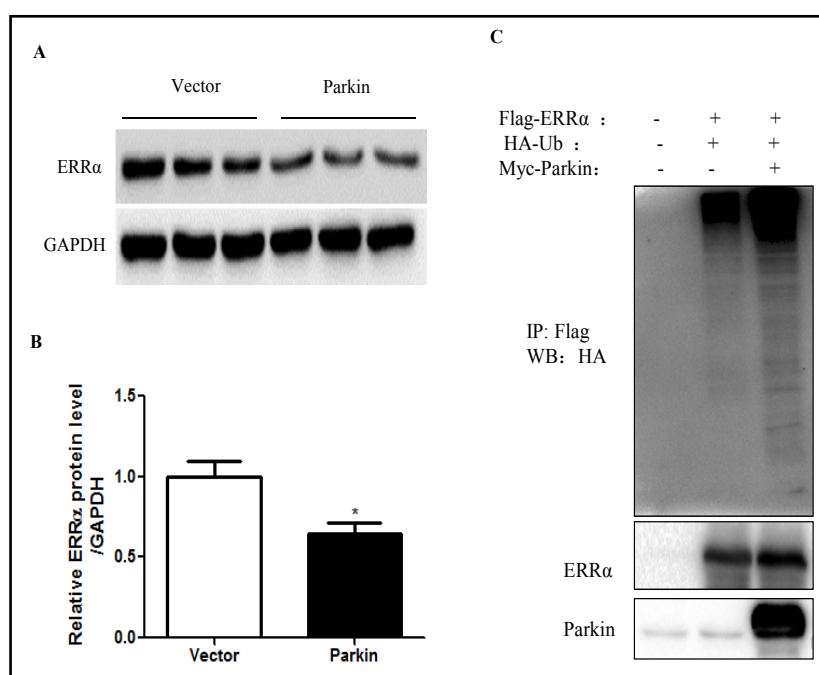
Fig. 1. Role of parkin in regulating the expressions of total-eNOS and p-eNOS in endothelial cells. (A) Mouse aortic endothelial cells were transfected with parkin plasmids or control vectors. Parkin, total-eNOS, and p-eNOS were examined by Western blotting. (B) Densitometric analyses of parkin, total-eNOS, and p-eNOS Western blots. (C) Densitometric analysis of p-eNOS relative to total-eNOS. (D) Mouse aortic endothelial cells were transfected with parkin siRNA or negative siRNA control. (E) Densitometric analyses of parkin, total-eNOS, and p-eNOS Western blots. (F) Densitometric analysis of p-eNOS relative to total-eNOS. The values represent the means \pm SEM (n=3 in each group). * $p < 0.05$ vs. Control group. ** $p < 0.01$ vs. Control group. *** $p < 0.001$ vs. Control group.

p-eNOS in MAECs, Western blotting was used to analyze total and phosphorylated eNOS in MAECs transfected with parkin plasmids or parkin siRNA. Notably, parkin overexpression downregulated the expressions of total-eNOS and p-eNOS in MAECs (Fig. 1A & B). In contrast, parkin silencing enhanced the total-eNOS and p-eNOS in MAECs (Fig. 1D & E), indicating an important role of parkin in regulating eNOS in endothelial cells. However, parkin overexpression or silencing did not affect the ratio of p-eNOS to total-eNOS (Fig. 1C & F).

Parkin overexpression reduced $ERR\alpha$ protein expression and enhanced $ERR\alpha$ ubiquitination in mouse aortic endothelial cells

As a transcription factor, $ERR\alpha$ has been reported to regulate eNOS expression via direct binding to the promoter of eNOS gene [18]. Thus, we examined the effect of parkin overexpression on $ERR\alpha$ expression in MAECs. As expected, parkin overexpression significantly decreased $ERR\alpha$ expression in MAECs (Fig. 2A & B). Parkin is an E3 ligase, therefore, we speculated that parkin might increase the ubiquitination of $ERR\alpha$ in MAECs. To test this hypothesis, we co-transfected flag- $ERR\alpha$, HA-Ub, and Myc-Parkin in MAECs. The result showed that parkin overexpression enhanced the ubiquitination of $ERR\alpha$ (Fig. 2C). These data suggested that parkin overexpression downregulated $ERR\alpha$ in endothelial cells possibly by enhancing the ubiquitination of $ERR\alpha$ to some extent.

Fig. 2. Role of parkin overexpression in regulating ERR α expression and ubiquitination in endothelial cells. Mouse aortic endothelial cells were transfected with ERR α plasmids or control vectors. (A) Western blotting analysis of ERR α . (B) Densitometric analysis of ERR α Western blots. (C) Mouse aortic endothelial cells were co-transfected with Myc-parkin, HA-Ub, and flag-ERR α plasmids. Then proteins were immunoprecipitated with Flag antibody, and



ERR α ubiquitination was determined by Western blotting. The values represent the means \pm SEM (n=3 in each group). * p<0.05 vs. Control group.

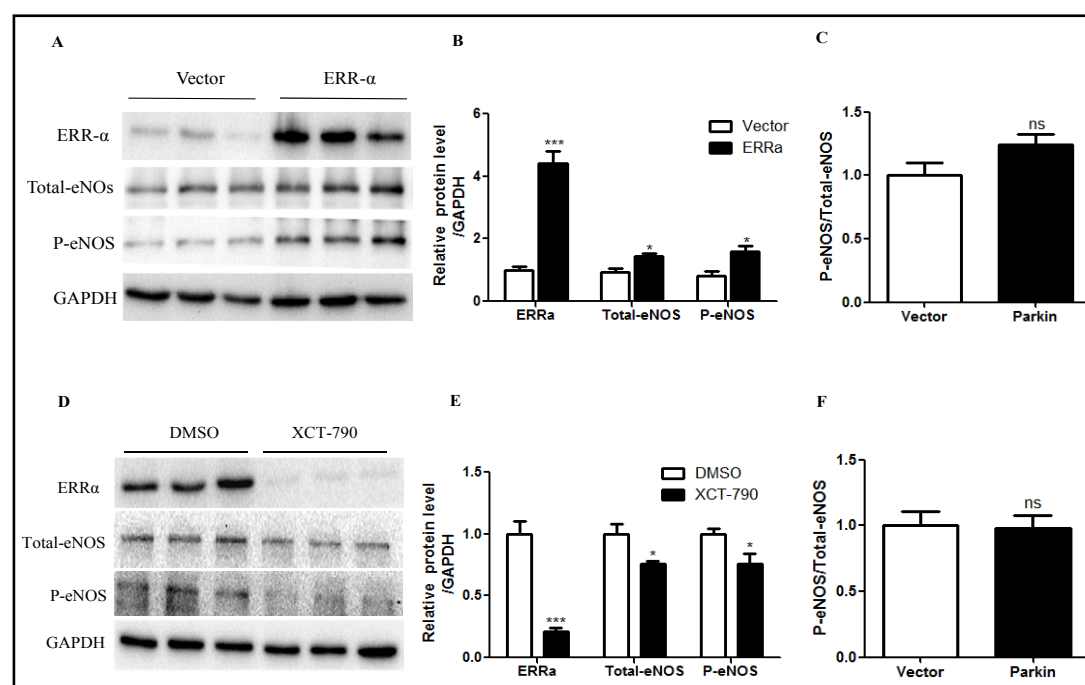


Fig. 3. ERR α regulated the expression of eNOS in endothelial cells. (A) Mouse aortic endothelial cells were transfected with ERR α plasmids or control vectors. Expressions of ERR α , total-eNOS, and p-eNOS were examined by Western blotting. (B) Densitometric analyses of ERR α , total-eNOS, and p-eNOS Western blots. (C) Densitometric analysis of p-eNOS relative to total-eNOS. (D) Mouse aortic endothelial cells were treated with ERR α inhibitor XCT-790 (10 μ M) or DMSO. Expressions of ERR α , total-eNOS, and p-eNOS were examined by Western blotting. (E) Densitometric analyses of ERR α , total-eNOS, and p-eNOS Western blots. (F) Densitometric analysis of p-eNOS relative to total-eNOS. The values represent the means \pm SEM (n=3 in each group). * p<0.05 vs. Control group. *** p<0.001 vs. Control group.

ERR α regulated the expression of eNOS in mouse aortic endothelial cells

To define the role of $ERR\alpha$ in modulating eNOS, $ERR\alpha$ was overexpressed in MAECs. As shown by the data, $ERR\alpha$ overexpression enhanced the expressions of total-eNOS and p-eNOS (Fig. 3A & B). In contrast, a specific $ERR\alpha$ inhibitor XCT-790 (10 μ M) decreased both total- and p-eNOS in MAECs with no effect on the ratio of p-eNOS to total eNOS (Fig. 3C-F). These data demonstrated a direct action of $ERR\alpha$ in regulating eNOS expression in MAECs.

Parkin overexpression-caused eNOS downregulation was independent of the autophagy in mouse aortic endothelial cells

As an E3 ligase, parkin plays a known role in modulating mitochondrial homeostasis and mitophagy. Thus, MAECs were transfected with parkin plasmids or empty vectors for 24 h. Significantly, as shown in Fig. 4, overexpression of parkin not only decreased MMP but also enhanced ROS production, indicating a mitochondrial dysfunction. Furthermore,

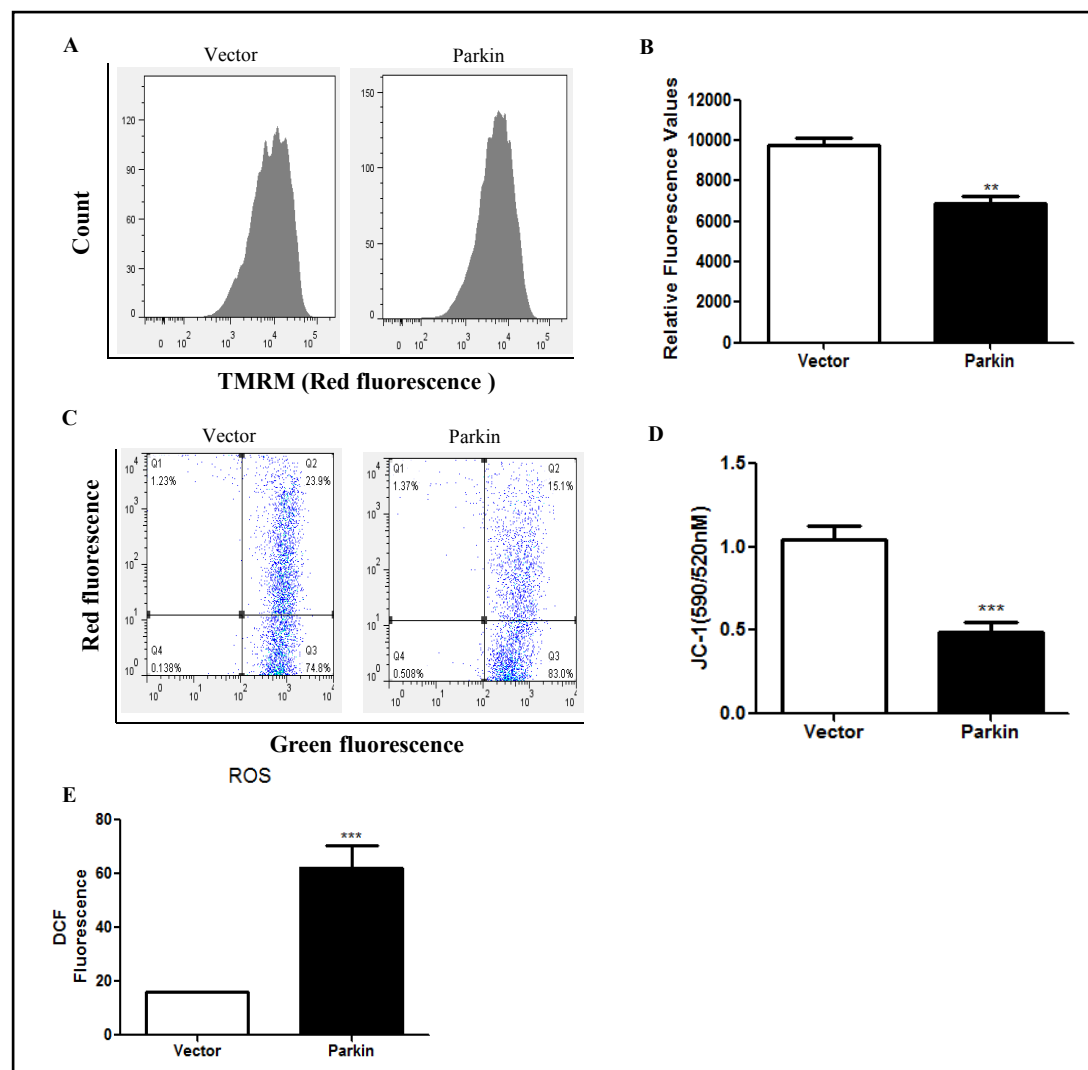


Fig. 4. Parkin overexpression induced mitochondrial dysfunction in endothelial cells. Mouse aortic endothelial cells were transfected with parkin plasmids or control vectors. (A & C) Mitochondrial membrane potential was determined by TMRM and JC-1 fluorescence dye. (B & D) Quantitative analysis of mitochondrial membrane potential. (E) ROS was determined by DCF fluorescence. The values represent the means \pm SEM (n=3 in each group). ** p<0.01 vs. Control group. *** p<0.001 vs. Control group.

Fig. 5. Effect of parkin overexpression on the expressions of LC3-II, P62, and TOM 20 in endothelial cells. Mouse aortic endothelial cells were transfected with parkin plasmids or control vectors. (A) Western blotting analyses of parkin and LC3-II. (B) Densitometric analyses of parkin and LC3-II Western blots. (C) Western blotting analyses of parkin, TOM 20, and P62. (D) Densitometric analyses of parkin, TOM 20, and P62 Western blots. The values represent the means \pm SEM (n=3 in each group). * p<0.05 vs. Control group. ** p<0.01 vs. Control group. *** p<0.01 vs. Control group.

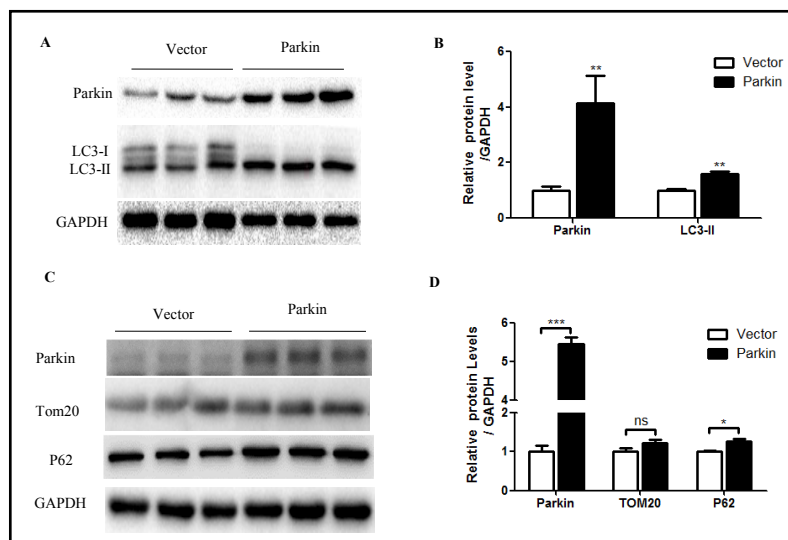
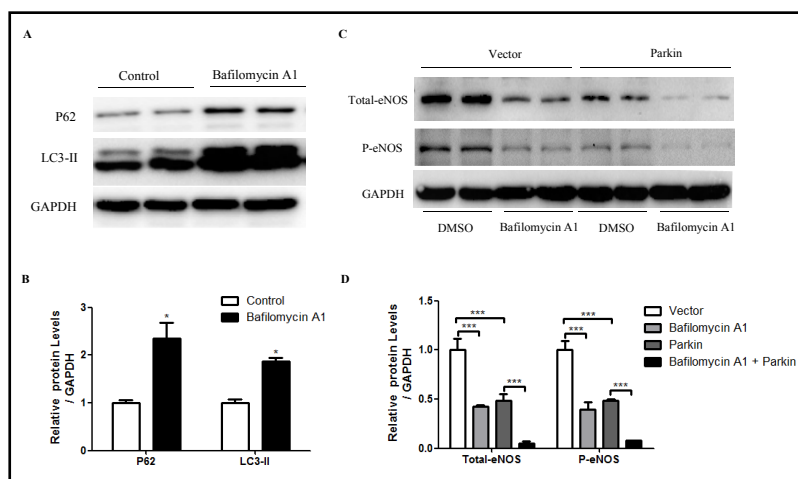


Fig. 6. Parkin overexpression-induced reduction of eNOS was independent of autophagy in endothelial cells. (A) Mouse aortic endothelial cells were treated with autophagy inhibitor bafilomycin A1 for 24 hours, then LC3-II and P62 were determined by Western blotting. (B) Densitometric analyses of LC3-II and P62 Western blots. (C) Mouse aortic endothelial cells were pre-treated with autophagy inhibitor bafilomycin A1 for 2 hours and then transfected with parkin plasmids or control vectors. Total-eNOS and p-eNOS were examined by Western blotting. (D) Densitometric analyses of total-eNOS and p-eNOS Western blots. The values represent the means \pm SEM (n=3 in each group). * p<0.05 vs. Control group. *** p<0.001 vs. Control group.



autophagy/mitophagy markers of LC3-II and P62, and mitochondrial protein TOM 20 were also examined by Western blotting. As shown by the data, parkin overexpression enhanced both LC3-II and p62 with no effect on TOM 20 expression (Fig. 5A-D), suggesting an impaired mitophagy in mouse aortic endothelial cells. Then we used autophagy inhibitor bafilomycin A1 to treat cells before parkin plasmid transfection. As shown in Fig. 6A-B, bafilomycin A1 enhanced both LC3-II and p62, indicating a blockade of autophagy. Moreover, bafilomycin A1 and parkin overexpression all decreased the expressions of total-eNOS and p-eNOS, while bafilomycin A1 further strikingly downregulated eNOS expression in cells with parkin overexpression (Fig. 6C-D), suggesting that parkin overexpression-induced eNOS reduction might be independent of impaired autophagy.

Parkin overexpression-caused eNOS downregulation was independent of the apoptosis in mouse aortic endothelial cells

To further examine the effect of parkin overexpression on the fate of endothelial cells, cell apoptosis was examined in MAECs transfected with parkin plasmids or empty vectors. Consistent with the impaired mitochondrial function, overexpression of parkin significantly increased cell apoptosis (Fig. 7A-B). Interestingly, when cells were treated with autophagy inhibitor 3-MA along with parkin overexpression, the cell apoptosis was further enhanced by a similar degree shown in cells with 3-MA alone treatment, suggesting that parkin overexpression triggered apoptotic response independently of autophagy in MAECs. Furthermore, an apoptosis initiator carbonyl cyanide m-chlorophenylhydrazone (CCCP) treatment strikingly increased cleaved-caspase-3 without affecting the expressions of ERR α and eNOS (Fig. 8A). Consistently, when cells were treated with caspase inhibitor Z-VAD-FMK before parkin overexpression, eNOS expression was also unaffected (Fig. 8B-D). These data suggested that the reduction of eNOS and ERR α in endothelial cells with parkin overexpression could be independent of cell apoptosis.

Parkin overexpression led to the apoptosis and mitochondrial dysfunction in HL-1 cells

Lastly, we examined the effect of parkin overexpression on apoptotic response and MMP in HL-1 cells. Similarly, overexpression of parkin significantly increased cell apoptosis and decreased mitochondrial membrane potential in HL-1 cells (Fig. 9A-D), indicating a detrimental role of parkin overexpression in HL-1 cells.

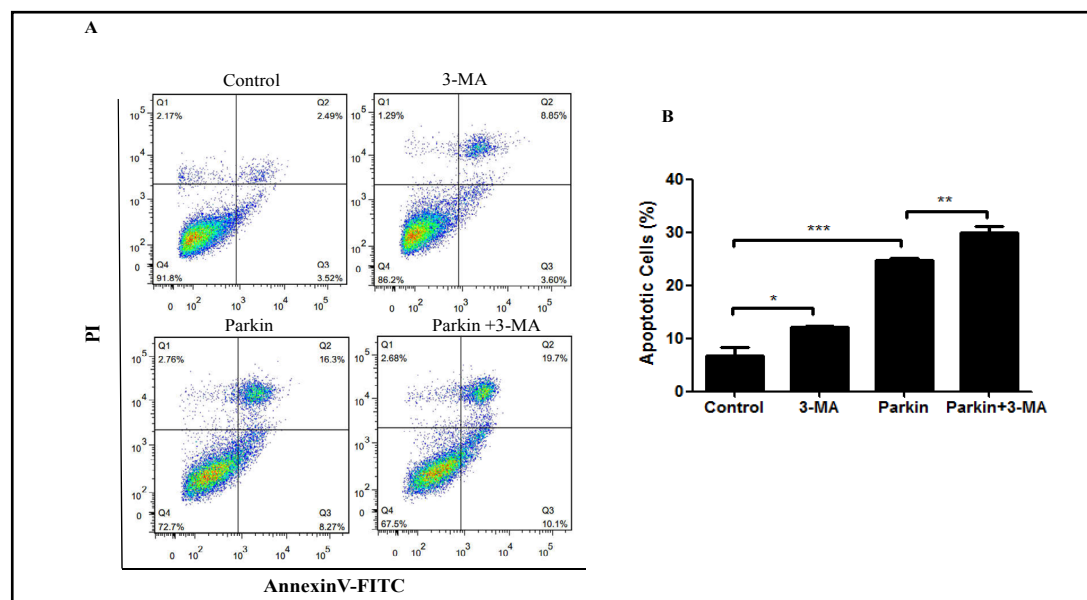


Fig. 7. Parkin overexpression-induced apoptosis in endothelial cells was independent of autophagic response. Mouse aortic endothelial cells were pretreated with autophagy inhibitor 3-MA and then transfected with parkin plasmids or control vectors. The apoptosis of mouse aortic endothelial cells was assessed by Annexin-V/PI staining. (A) Representative images of flow cytometry. (B) Quantification of the percentage of apoptotic cells. The values represent the means \pm SEM (n=3 in each group). * p<0.05 vs. Control group. ** p<0.01 vs. Control group. *** p<0.001 vs. Control group.

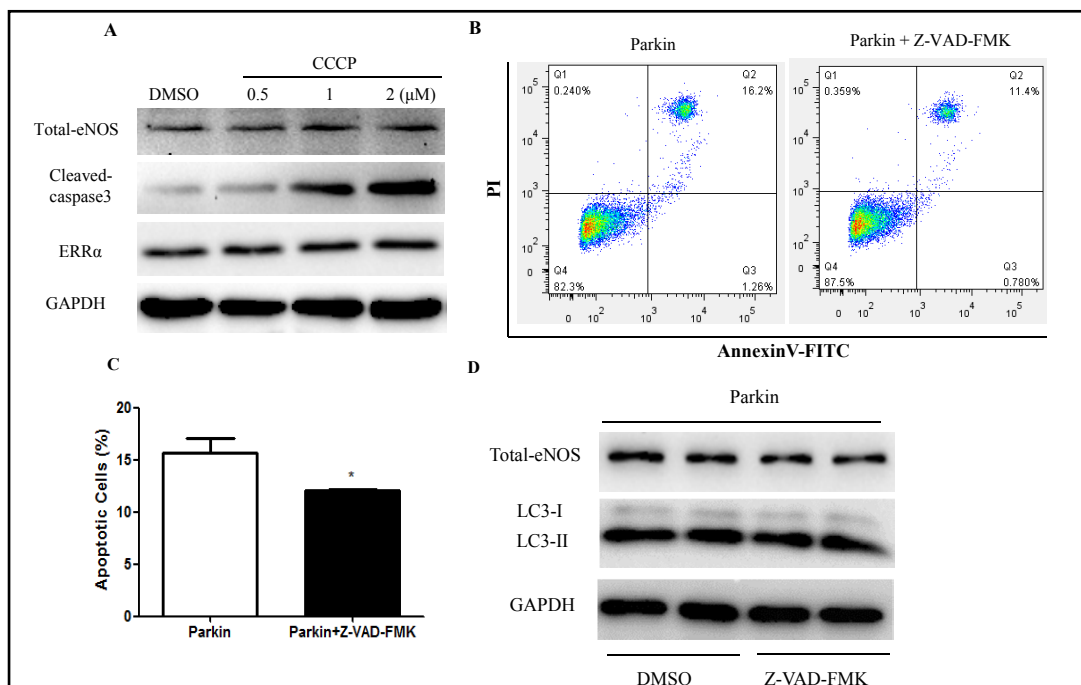
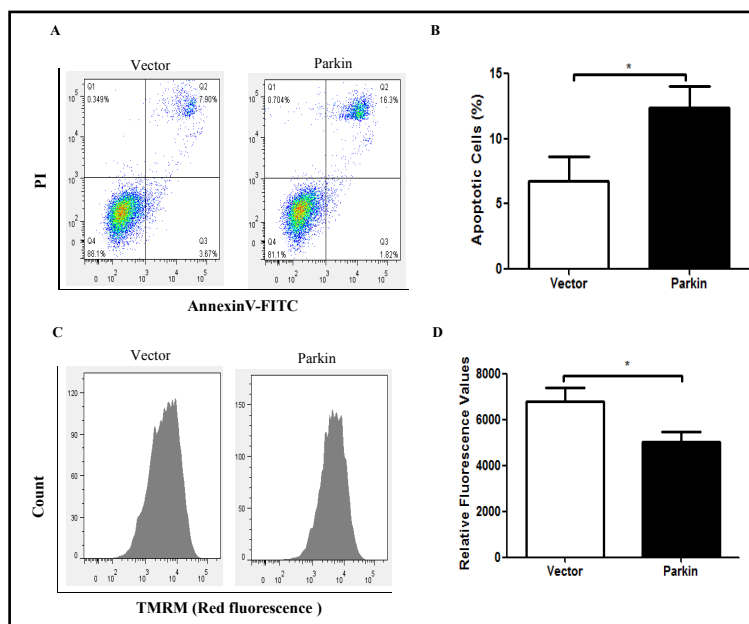


Fig. 8. Parkin overexpression-induced decrement of eNOS in endothelial cells was independent of cell apoptosis. (A) Mouse aortic endothelial cells were treated with indicated doses of CCCP (0, 0.5, 1, 2 μ M). Total-eNOS, cleaved-caspase-3, and $ERR\alpha$ were examined by Western blotting. (B & C) Mouse aortic endothelial cells were pretreated with caspase inhibitor Z-VAD-FMK for 2 hours and then transfected with parkin plasmids. The apoptosis of mouse aortic endothelial cells was assessed by Annexin-V/PI staining. (D) Western blotting analyses of total-eNOS and LC3-II. The values represent the means \pm SEM (n=3 in each group). * p<0.01 vs. Parkin group.

Fig. 9. Parkin overexpression caused apoptosis and decreased mitochondrial membrane potential in HL-1 cells. HL-1 cells were transfected with parkin plasmids or control vectors. (A & B) The apoptosis of HL-1 cells was assessed by Annexin-V/PI staining. (C & D) Mitochondrial membrane potential was determined by using TMEM. The values represent the means \pm SEM (n=3 in each group). * p<0.05 vs. Control group.



Discussion

As an E3 ubiquitin ligase, parkin is of importance in maintaining endothelial integrity and mitochondrial function by ubiquitinating unfolded and damaged proteins [20]. Previous findings demonstrated that parkin was upregulated in the vascular wall of obese, diabetic and atherosclerotic mice, and parkin-related mitophagy is protective in these disease models [21]. Thus, overexpression or activation of parkin potentially serves as a strategy in treating vascular diseases. However, the role of parkin overexpression in normal endothelial cells is uncertain. In the present study, we examined the role of parkin overexpression in vascular endothelial cells. Our findings indicated that parkin overexpression led to a decrement of total-eNOS and p-eNOS. Endothelial NO synthase (eNOS) catalyzes L-arginine to produce nitric oxide (NO) in vascular endothelial cells. NO has been documented as a critical factor in control of blood pressure, blood flow, and the pathogenesis of atherosclerosis [22, 23]. It is known that the impairment of eNOS/NO signaling is a hallmark of endothelial dysfunction [4]. Thus, our findings suggested that parkin overexpression might cause endothelial dysfunction in normal blood vessels.

Estrogen related receptor α (ERR α) is an orphan nuclear receptor and regulates energy metabolism and mitochondrial biogenesis [24, 25]. ERR α and its co-activator PGC-1 α work with each other to affect the transcription of target genes [16, 17]. In 2003, Sumi D and Ignarro LJ reported that ERR α could directly regulate the expression of eNOS via a transcriptional mechanism [18]. A recent study also showed that endothelial PGC-1 α protected against angiotensin II-induced hypertension *in vivo* possibly through activating ERR α /eNOS axis [26]. In the current study, we demonstrated that parkin overexpression decreased the expression of ERR α . Since parkin is an E3 ligase, we speculated that parkin might affect the ubiquitination of ERR α . As expected, our results showed that parkin overexpression increased the ubiquitination of ERR α . In agreement with our findings, other group also reported that parkin could degrade ERR α by a ubiquitination mechanism in SH-SY5Y cells [19]. Consistently, ERR α inhibition by XCT-790 reduced total-eNOS and p-eNOS, while ERR α overexpression enhanced both. These data suggested that parkin overexpression-induced downregulation of eNOS was possibly through an ERR α -mediated mechanism.

Mitochondria play crucial roles in energy metabolism, signal transduction, and cell viability, and are responsible for the production of more than 95% reactive oxygen species (ROS). Mitochondrial membrane potential (MMP) disruption is not only a marker of mitochondrial dysfunction but also a contributor of apoptotic response [27]. In the present study, parkin overexpression reduced mitochondrial membrane potential and enhanced ROS production in MAECs, indicating an impairment of mitochondrial function.

Impaired autophagy may cause severe clinical complications such as cancers, neurodegenerative diseases, cardiac diseases, and kidney diseases [28-31]. A lot of preclinical studies suggested that autophagy could be a double-edged sword in many pathological conditions including cardiovascular diseases [28, 32-36]. Autophagy/mitophagy has been a hot target for the treatment of diseases including cardiovascular disease. In order to define the role of parkin overexpression in autophagy in our experimental setting, we detected the autophagy/mitophagy markers of LC3-II and p62 and mitochondrial protein TOM 20. The data demonstrated an impaired autophagy in endothelial cells with parkin overexpression. Furthermore, we used autophagy inhibitor bafilomycin A1 to treat cells with parkin overexpression. As expected, bafilomycin A1 further reduced eNOS levels after parkin overexpression, suggesting that parkin overexpression-caused eNOS downregulation could be independent of impaired autophagy.

Furthermore, we observed that parkin overexpression induced apoptosis in MAECs, suggesting a proapoptotic role of parkin overexpression in the present experimental setting. Interestingly, when we treated endothelial cells with autophagy inhibitor 3-MA, we found a similar increment of cell apoptosis in MAECs with or without parkin overexpression, indicating that parkin overexpression-induced cell apoptosis could be independent of

impaired autophagy. Then endothelial cells were treated with an apoptosis initiator CCCP, which did not affect the expressions of eNOS and $ERR\alpha$. In agreement with this result, caspase inhibitor Z-VAD-FMK did not affect eNOS expression in endothelial cells with parkin overexpression. Overall, these data indicated that parkin overexpression-caused dysregulation of $ERR\alpha$ /eNOS axis should be independent of apoptotic response in MAECs.

To further extend our findings to other cells, we finally examined the role of parkin overexpression in cardiac HL-1 cell line. Similarly, overexpression of parkin in HL-1 cells significantly increased cell apoptosis and decreased mitochondrial membrane potential, indicating that parkin overexpression was also detrimental in normal cardiomyocytes.

Conclusion

Employing the genetic and pharmacological approaches, we found that parkin overexpression reduced eNOS possibly through an $ERR\alpha$ ubiquitination-associated mechanism. These data also suggested that a systemic overexpression/activation of parkin might be detrimental in vessels with normal endothelium, although it might be protective in the lesion regions of vessels.

Acknowledgements

This work was supported by Grants from the National Natural Science Foundation of China (nos. 81600352, 81670647, 81600557, and 81570616), the National Key Research and Development Program (no. 2016YFC0906103), Natural Science Foundation of Jiangsu Province (no. BK20160137) and Nanjing Medical University (2015NJMUZD053).

Disclosure Statement

The authors declare that there are no conflicts of interest.

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