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XPO1-mediated nuclear export of RNF146 protects from angiotensin II-induced endothelial cellular injury

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ABSTRACT

Endothelial cells death induced by angiotensin II (Ang II) plays a role in vascular injury. RNF146 is identified as a E3 ubiquitin ligase, which promotes cell survival under many types of stresses. However, the role of RNF146 in endothelial cellular injury is unknown. In human umbilical vein endothelial cells (HUVECs), Ang II treatment led to cell death by oxidative stress and promoted RNF146 to accumulate in nucleus in time dependent manner. Nuclear export signal was found in the RNF146's sequence. The interaction between RNF146 and XPO1 was further confirmed by co-immunoprecipitation. Inhibition of XPO1 with KPT-185 increased the level of RNF146 in nucleus. The expression of XPO1 was suppressed responding to Ang II treatment. Overexpression of XPO1 facilitated the nuclear shuttling of RNF146, which protected from Ang II-induced cell death. Moreover, overexpression of RNF146 in HUVECs reduced the cell death induced by Ang II, whereas inhibition of XPO1 abolished the protective effect of RNF146. Therefore, our data demonstrated that RNF146 was a protective factor against cell death induced by AngII in human endothelial cells, which was dependent on XPO1-mediated nuclear export.

1. Introduction

Endothelial cells (ECs) play a critical role in maintaining the integrity and tone of vascular wall. The malfunction of ECs contribute to vascular injury, which leads to development or progression of various vascular disease, including atherosclerosis, restenosis and hypertension [1,2]. Increasing of oxidative burden induced by angiotensin II (Ang II) severed as major trigger of endothelial cell death, which is critically involved in the vascular pathology remodeling [3,4]. Thus, further efforts to elucidate the mechanisms, how Ang II acts as a mediator of endothelial cell death would yield novel therapeutic approach for cardiovascular disorders.

The RING-domain E3 ubiquitin ligase RNF146 recognizes the poly-ADP-ribosylated substrates by its Trp—Trp—Glu (WWE) domain and targets them for proteasome degradation [5,6]. RNF146 showed protective effect in hippocampal neuronal cells by inhibiting oxidative stress-induced cell death [7]. In cardiac myocytes, RNF146 also exerts protection against oxidant-induced cell death [8]. RNF146 translocated to the nucleus responding to oxidative-stress induced cellular injury to trigger the exit of the cell death-effector, Poly (ADP-ribose) polymerase-1 (PARP-1), for degradation [8]. Furthermore, RNF146 was reported to inhibit PARP-1-dependent cell death by decreasing apoptosis inducing factor (AIF) nuclear translocation in immature brain subjected to hypoxic—ischemic damage [9]. Hence, the main function of RNF146 is to target the poly-ADP-ribosylated cell death-effector and prompt their translocation from nuclear to cytoplasm for proteasome-dependent degradation. However, the role of RNF46 in EC dysfunction has not been identified so far.

Exportin 1 (XPO1), also known as chromosome region maintenance 1 (CRM1), is one member of nuclear export receptors, which mediates the nuclear-cytoplasmic partitioning of variety proteins and certain RNAs by recognizing leucine-rich nuclear export signals (NES) [10,11]. To date, many tumor suppressors and anti-apoptotic regulators, such as P53, BRCA1, STAT3, and survivin, were identified as the cargos interacting with XPO1 [12–14]. The appropriate nuclear-to-cytoplasmic translocation of the essential tumor suppressors mediated by XPO1 plays roles in cancer initiation and

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malignancies by regulating cell-cycle transition, the DNA damage response, apoptosis, and autophagy. Recently, XPO1-dependent gene interaction network was predicted to be one of the key modules in EC dysfunction [15].

In this study, we identified E3 ubiquitin ligase RNF146 as a novel cargo of XPO1-mediated nuclear export in human umbilical vein endothelial cells (HUVECs). The amount of cytoplasmic RNF146 that was dependent on the XPO1 played a protective role against Ang II-induced HUVECs injury by promoting AIF degradation. Inhibition of XPO1 abrogated this protective effect by facilitating RNF146 accumulation in nucleus.

2. Materials and methods

2.1. Cell culture and reagents

HUVECs were obtained from Type Culture Collection Committee of Chinese Academy of Science (Shanghai, China), cultured in Dulbecco's modified Eagle's medium (Gibco), 10% fetal bovine serum (Gibco), and maintained in a humidified incubator containing 5% CO_2 at 37 °C. The culture medium was replaced every 48 h (h). KPT-185 was purchased from Selleck Chemicals and dissolved in Dimethylsulfoxide (DMSO). Angiotensin II was purchased from Sigma-Aldrich and dissolved in sterile, ultrapure water.

2.2. Plasmids preparation and transfection

Full-length XPO1 and RNF146 cDNA were amplified from a human cDNA library using standard PCR techniques and subcloned into p3xFLAG-CMV (Sigma) and pcDNA3.1-Myc-His vector, respectively (Invitrogen). The sequences of both constructs used were confirmed by direct sequencing. HUVECs were transfected with the plasmids by Lipofectamine® 2000 regent according to manufacturer's instructions.

2.3. Cellular Reactive Oxygen Species (ROS) detection

ROS was measured with Cellular Reactive Oxygen Species Detection Assay Kit (Abcam, ab113851). Briefly, one day before detection, HUVECs were cultured in a 96-well plate. After treated with Ang II, HUVECs were collected and washed with PBS. Then the cells were stained with 20 μM 2′,7′−dichlorofluorescin diacetate (DCFDA) in serum-free DMEM for 30 min at 37 °C。After that, fluorescence was measured by the fluorospectrophotometer (excitation wavelength 485 nm and emission wavelength 535 nm).

2.4. Lactate dehydrogenase (LDH) assay

Cellular injury was determined by measurement of LDH, a cytoplasmic enzyme released from cells into the culture medium. LDH in the culture medium was detected using the LDH Cytotoxicity Assay Kit (Thermo Scientific) according to the manufacturer's instructions. Briefly, 50 μL of supernatant from each well was collected and incubated with Reaction Mixture for 30 min at room temperature. Then the reaction was stopped by adding Stop Solution. The activity of LDH was calculated from the subtraction the 680 nm absorbance value (background signal from instrument) from the 490 nm absorbance [(LDH at 490 nm) - (LDH at 680 nm)]. The results were normalized to the maximal LDH release, which was determined by treating control wells for 45 min with Lysis Buffer to lyse all cells.

2.5. Nuclear−cytoplasmic fractionation, co-immunoprecipitation and western blot

Nuclear−cytoplasmic fractionation separation was performed using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Fisher scientific) according to the manufacturer's protocol. Total proteins were extracted from HUVECs with RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific) containing Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific). Cell lysates were cleared by centrifugation at 12,000 g for 10 min at 4 °C. For immunoprecipitation, the products were collected on Protein A/G PLUS-Agarose (Santa Cruz biotechnology). For Western Blot, proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (EMD Millipore). The PVDF membranes were blocked by incubating with 5% non-fat milk for 1 h at room temperature, and then incubated with antibodies against

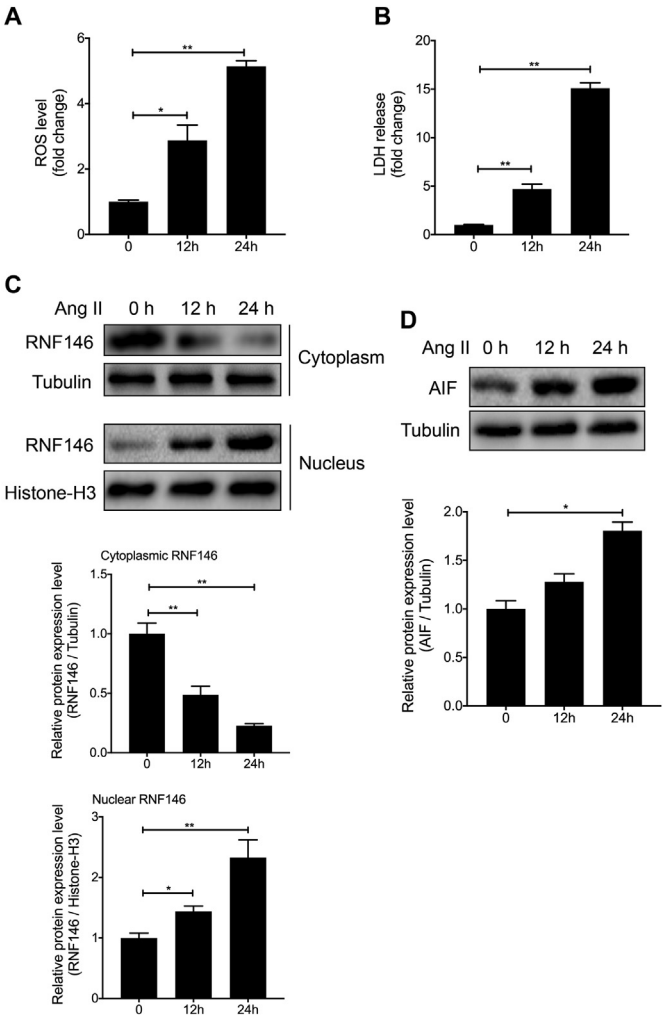

Fig. 1. Ang II induced HUVECs death and cytoplasmic-to-nuclear translocation of RNF146. (A) Intracellular ROS level assay in HUVECs after Ang II (200 nM) treatment for 12 h and 24 h. (B) Measurement of LDH release level from HUVECs after Ang II treatment for 12 h and 24 h. (C) Western blot analysis of RNF146 in cytoplasmic fraction and nuclear fraction after Ang II treatment for 24 h. Tubulin and Histone-H3 were loading controls for cytoplasmic and nuclear fraction, respectively. (D) Western blot analysis of AIF in HUVECs induced by Ang II treatment. Data represented mean ± s.e.m., n = 5; *P < 0.05, **P < 0.01. ROS, Reactive Oxygen Species; HUVECs, human umbilical vein endothelial cells; Ang II, angiotensin II; LDH, Lactate dehydrogenase.

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XPO1 (Abcam, ab180144), RNF46 (Abcam, ab201212), Apoptosis-inducing factor (AIF) (proteintech, 17984-1-AP), alpha-Tubulin (proteintech, 11224-1-AP) and Histone-H3 (proteintech, 17168-1-AP) at 4 °C overnight. The PVDF membranes were incubated with Horseradish peroxidase (HRP)-conjugated IgG (proteintech) as secondary antibody for 1 h at room temperature. The images of blots were quantified and analyzed using the ImageJ software.

2.6. Statistical analysis

All data were expressed as mean ± SEM of at least three independent experiments. Significant differences between groups were assessed by univariate ANOVA (more than two groups) or unpaired *t*-test (two groups). For all tests, a value of $P < 0.05$ was considered statistically significant. GraphPad Prism software was used for calculation of all statistics of significance.

3. Results

3.1. Ang II led to cellular injury by ROS generation and promoted nuclear accumulation of RNF146 in HUVECs

HUVECs was treated by Ang II (200 nM) for the time course (0 h, 12 h, and 24 h) to induce cellular injury, and LDH release level was measured as the marker of cell death. Treatment with Ang II induced a significant increase of both ROS level (Fig. 1A) and LDH release level in a time dependent manner (Fig. 1B). Then we detected the expression of RNF146 in nuclear and cytoplasm by western blot analysis, respectively. The results indicated that the amount of cytoplasmic RNF146 decreased, whereas the nuclear RNF146 increased after Ang II stimuli (Fig. 1C). Apoptosis inducing factor (AIF) that promotes DNA damage and cell death in response to oxidative stress has been recently reported to be ubiquitinated

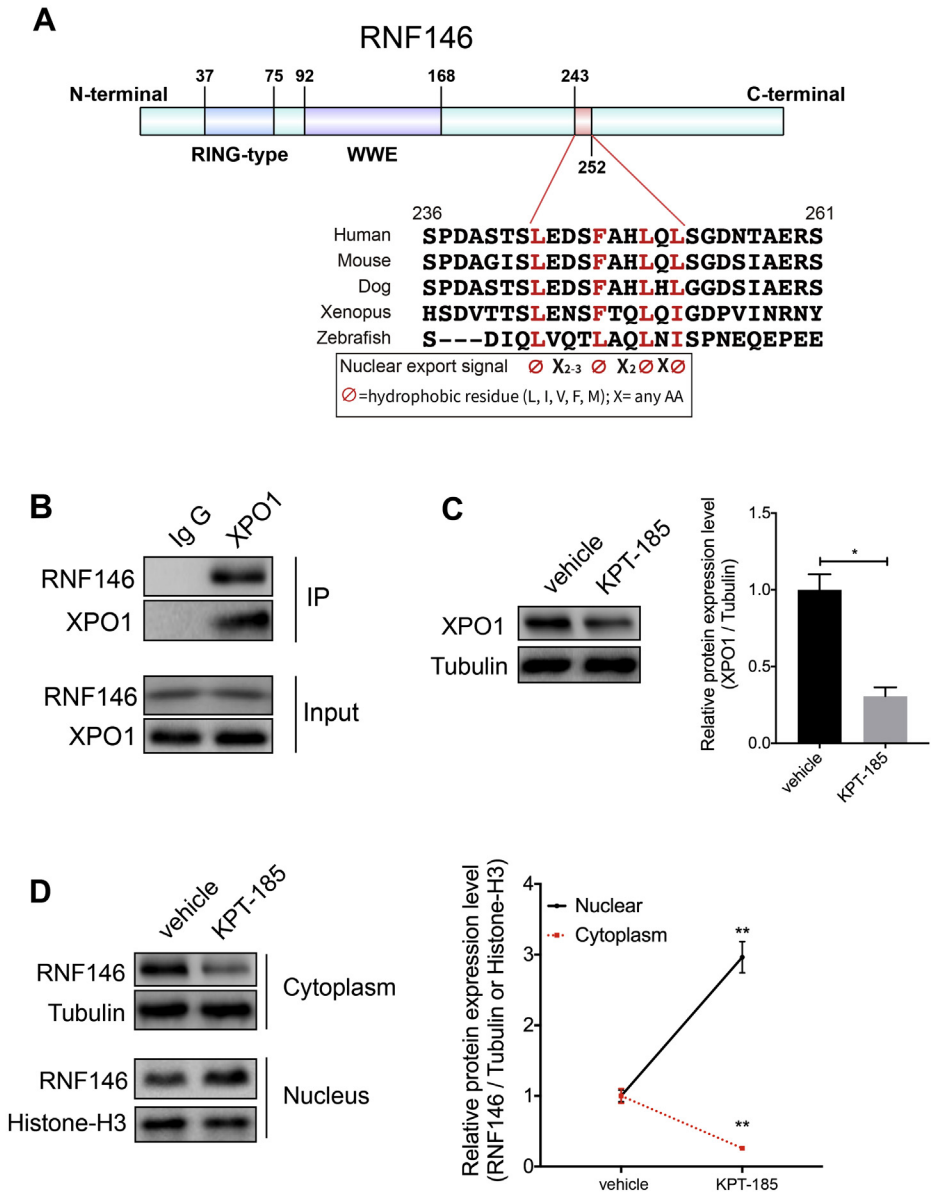

Fig. 2. The subcellular localization of RNF146 is regulated by XPO1-mediated nuclear export. (A) Sequence alignment of NES between the human, mouse, dog, xenopus, and zebrafish RNF146 proteins. The NES was highlighted with red color. (B) Immunoprecipitated RNF146 with control IgG or the antibody to XPO1. (C) Western blot analysis of XPO1 expression in HUVECs exposed to 50 nM KPT-185. (D) Western blot analysis of RNF146 in cytoplasmic and nuclear fraction after KPT-185 treatment. Data represented mean ± s.e.m., n = 5; *P < 0.05, **P < 0.01. NES, nuclear export signal. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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and degraded by RNF146 [9]. Therefore, we evaluated the protein expression level of AIF after Ang II treatment, which showed that Ang II exposure promoted the accumulation of AIF in HUVECs (Fig. 1D). These data demonstrated that the Ang II led to cytoplasm-to-nuclear translocation of RNF146 and AIF accumulation, which might be involved in cell death upon oxidative stress in HUVECs.

3.2. The subcellular localization of RNF146 is regulated by XPO1-mediated nuclear export

A recent report had found RNF146 as one cargo candidates for XPO1-dependent nuclear export in cultured human cells [16]. As shown in Fig. 2A [17], we identified the leucine-rich nuclear export signal (NES) in the amino acids sequence of RNF146. And there was a high degree of homology of this NES between the human, mouse, dog, xenopus, and zebrafish RNF146 proteins. Then the co-immunoprecipitation assay confirmed the endogenous interaction between RNF146 and XPO1 in HUVECs (Fig. 2B). Next, a specific XPO1 inhibitor, KPT-185, was used to address whether XPO1 affected the localization of RNF146. We found that XPO1 protein expression was decreased with KPT-185 (50 nM) treatment for 24 h (Fig. 2C). Moreover, inhibition of XPO1 with KPT-185 treatment also induced the nuclear accumulation of RNF146 (Fig. 2D). Altogether, these data first revealed RNF146 as an endogenous target of XPO1-mediated nuclear export, which regulated the subcellular localization of RNF146.

3.3. Ang II-induced accumulation of RNF146 in nucleus is due to the loss of XPO1 expression level

We have shown that RNF146 was a cargo of XPO1-mediated nuclear export. Next, we tried to explore whether XPO1 contributed to the accumulation of RNF146 in nucleus in response to Ang II exposure. The results of western blot showed that the amount of XPO1 was reduced by nearly 50% after Ang II treatment (Fig. 3A). Then we rescued the expression of XPO1 by plasmids transfection to further determine the role of XPO1 in the distribution of RNF146 in cytoplasmic and nuclear components. Compared with the vector transfected group, transfection of Flag-XPO1 significantly elevated the XPO1 protein level (Fig. 3B). As shown in Fig. 3C, Ang II treatment promoted RNF146 translocation to the nucleus. However, XPO1 overexpression restored the amount of cytoplasmic RNF146 and reduced the level of nuclear RNF146 (Fig. 3C). In addition, we also observed that overexpression of XPO1 suppressed LDH release level against Ang II induced cell death (Fig. 3D). Collectively, our results revealed that accumulation of RNF146 in nucleus was caused by the insufficiency of XPO1 when HUVECs was subjected to Ang II induced cellular injury. By contrast, XPO1 recovery could facilitate nuclear export of RNF146 and attenuate the cellular injury.

3.4. The protection effect of RNF146 is dependent on XPO1-mediated nuclear export

RNF146 has previously been reported to play a protective role in neurons. Hence, we wonder whether RNF146 could protect HUVECs against Ang II-induced cellular injury and the role of XPO1 in this process. To address this issue, HUVECs were RNF146-overexpressed and concomitantly treated with KPT-185, which then were subjected to Ang II treatment. LDH release level detection demonstrated that overexpression of RNF146 attenuated Ang II-induced HUVECs injury (Fig. 4A). However, inhibition of XPO1 by KPT-185 treatment abrogates the protective effect of RNF146 overexpression (Fig. 4A). Western blot analysis showed that RNF146 overexpression increased RNF146 amount both in nuclear and cytoplasmic components, while KPT-185 promoted RNF146 accumulation in the cell nucleus (Fig. 4B–C). Being comparable to LDH release level, cytoplasmic amount of AIF was significantly downregulated by RNF146 overexpression, which was abolished by XPO inhibition with KPT-185 treatment (Fig. 4D). These results taken together indicated that cytoplasmic RNF146 had the

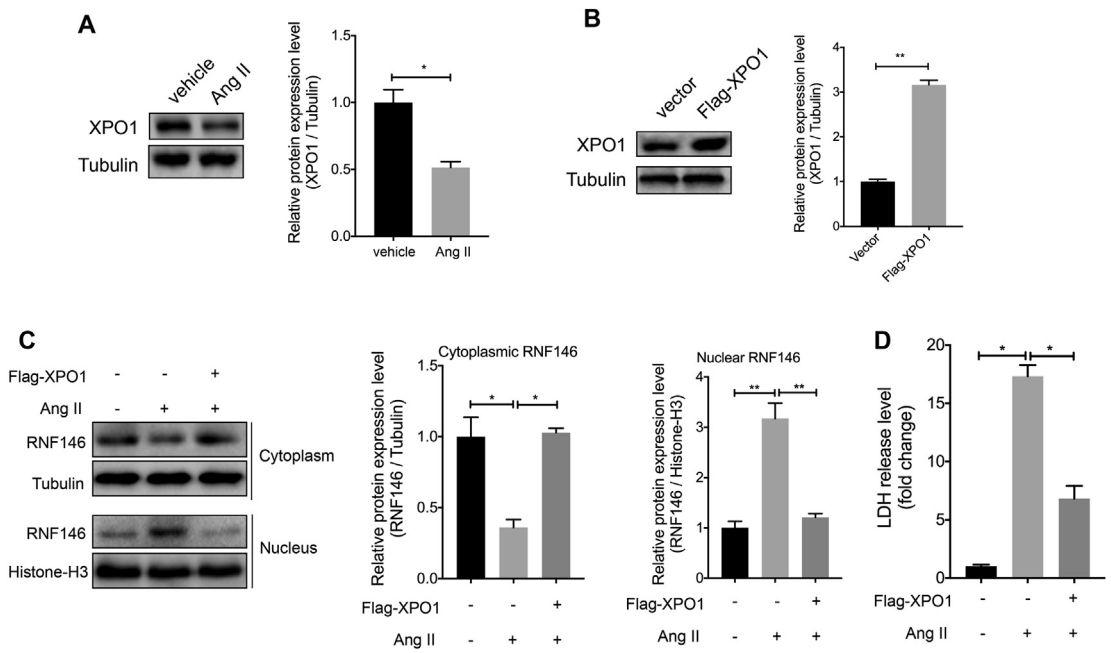

Fig. 3. Reduced XPO1 expression level contribute to nuclear accumulation of RNF146 in response to Ang II. (A) Western blot analysis of XPO1 expression responding to Ang II stimuli in HUVECs. (B) Western blot analysis of XPO1 expression after Flag-XPO1 plasmids transfection in HUVECs. (C) Western blot analysis of RNF146 in cytoplasmic and nuclear fraction after Ang II treatment in the presence of overexpressed XPO1. (D) LDH release measurement of HUVECs after Ang II treatment in the presence of overexpressed XPO1. Data represented mean ± s.e.m., n = 5; *P < 0.05, **P < 0.01.

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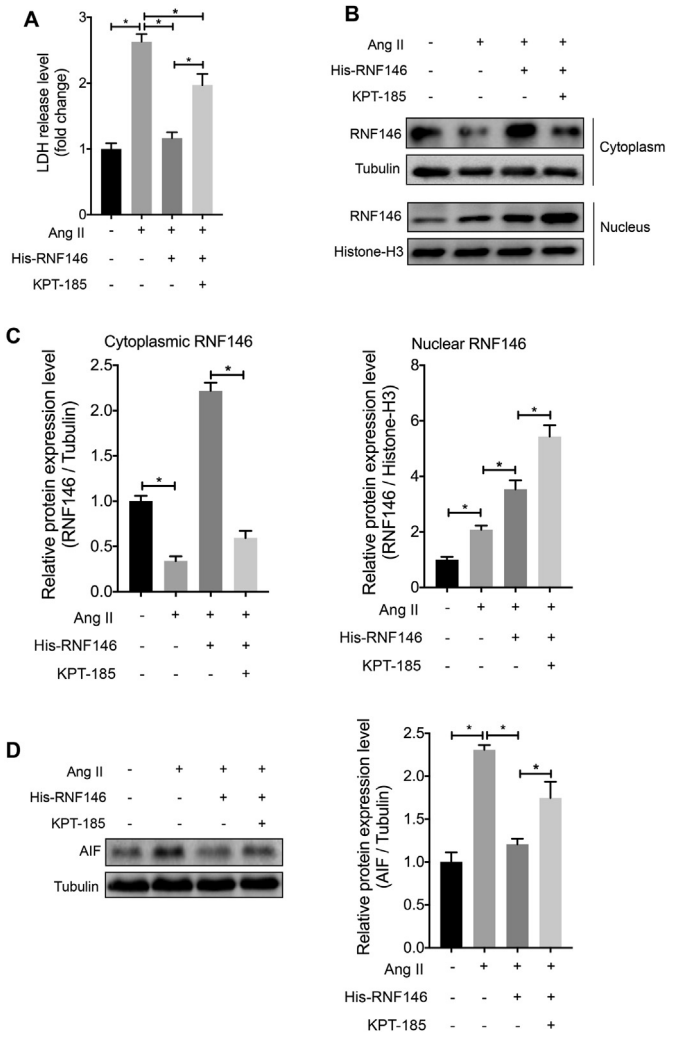

Fig. 4. The protection effect of RNF146 is dependent on XPO1-mediated nuclear export. (A) Measurement of LDH release level from Ang II-treated HUVECs after KPT-158 exposure in the presence of overexpressed RNF146. (B) Western blot analysis of RNF146 in cytoplasmic and nuclear fraction from Ang II-treated HUVECs after KPT-158 exposure in the presence of overexpressed RNF146. (C) Quantification of the data in **B**. (D) Western blot analysis of AIF in Ang II-treated HUVECs after KPT-158 exposure in the presence of overexpressed RNF146. Data represented mean ± s.e.m., n = 5; $^{*}P < 0.05$, $^{**}P < 0.01$.

protective effect against Ang II-induced HUVECs injury via enhancement of AIF degradation, which was dependent on XPO1-dependent nuclear export.

4. Discussion

The results we presented here suggested E3 ubiquitin ligase RNF146 as a protective factor against Ang II-induced cell death in HUVECs by repressing AIF protein expression. We also identified RNF146 as a novel cargo of XPO1-mediated nuclear export, and inhibition of XPO1 promoted the accumulation of RNF146 in nucleus. Additionally, the protective effect of RNF146 was dependent on normal function of XPO1-mediated nuclear export.

Ang II is an essential vasoactive substance that promotes vascular remolding. Endothelial cell damage and death caused by Ang II-elicited ROS generation play a key role in vascular remodeling [18]. Ang II-elicited oxidative stress provoke DNA damage via variant cell death signaling activation. Parthanatos, a distinct death pathway, is associated with the molecular events of activation of PARP-1, synthesis and accumulation of PAR polymer, and mitochondrial depolarization [19]. Much evidence revealed that PARP-1 expression could be induced by Ang II stimuli [20–22]. Moreover, inhibition of PARP-1 reduced Ang II-induced NF-κB activation in vascular endothelial cells [22]. AIF is recognized as a key cell death effector that mediates PARP-1-dependent cell death [23]. Many investigations suggested that PAR-mediated cell death is AIF-required [24]. Interestingly, previous studies showed that AIF could be induced and activated [25,26]. Our data also demonstrated Ang II treatment increased the expression of AIF in HUVECs, which implied PARP-AIF-mediated parthanatos play a role in Ang II-induced endothelial cellular injury.

In this study, RNF146 was found as a protective factor against Ang II-induced HEVECs death. Previous report has shown that RNF146 protects against cell death by suppressing oxidative stress-induced parthanatos, which implies that RNF146 is vital mediator of PARP-1 dependent pathway [7,8,27]. RNF146 has PAR-binding and E3 ligase activities. RNF146 can bind PARsylated substrates by its WWE domain and target them through ubiquitin proteasomal degradation [28,29]. AIF is a PAR-binding protein and that PAR binding effect is critical for translocation of AIF from the mitochondria to the nucleus [30]. RNF146 has been reported to serve as an E3 ubiquitin ligase of AIF, which promotes the degradation of cytoplasmic AIF and keeps AIF from translocating into the nucleus in neurons [27]. Indeed, in HUVECs, we also found overexpression of RNF146 downregulated the expression of AIF, which further confirmed that RNF146 protected against Ang II-induced cellular injury via mitigating AIF-dependent parthanatos.

XPO1-mediated nuclear export is responsible for appropriate distribution of its cargos. Overexpression of XPO1 has been observed in many types of cancers, which prevents the tumor-suppressors from cytoplasm-to-nucleus translocation [31]. Our results suggested that RNF146 was a cargo of XPO1, and nuclear export of RNF146 was governed by the expression of XPO1. RNF146 is a normally cytoplasmic protein and facilitate the ubiquitin mediated degradation of AIF and PARP-1 via proteasome in cytoplasm [8,27]. In addition, it can translocate to nucleus under oxidative stress or glutamate excitotoxicity [8,27]. In our study, it is also showed that loss of XPO1 contributed to accumulation of RNF146 in nucleus under cellular injury stress. Under this condition, we could not exclude the possibility of cytoplasm-to-nucleus translocation of RNF146 exerted its protective effect. However, our data showed that cytoplasmic RNF146 played a key role in its protective effect. Therefore, we suspected that XPO1-mediated RNF146 nuclear export was an essential process that was resistant to cytotoxicity. Indeed, RNF146 seems to be localized to the cytoplasm in the normal condition. Moreover, previous study showed that RNF146 promoted the degradation of cytoplasmic AIF and keeps AIF from translocating into the nucleus [27], which further suggested cytoplasmic RNF146 rather than nuclear RNF146 had protective effect against oxidative stress. In another hand, to mediate the cellular toxicity, PAR polymer exits from the nucleus [32], where RNF146 can capture PARylated proteins for their proteasomal degradation [8]. It has been reported that XPO1-mediated MDM2 nuclear export is responsible for the continual shuttling of p53 molecules from the nucleus to the cytoplasm, targeting it for degradation by cytoplasmic proteasomes [33]. Hence, we speculated that nuclear-entered RNF146 could interact with PARylated AIF in the nucleus, then promoted AIF to cytoplasmic proteasomal degradation via XPO1-mediated nuclear export, which might be confirmed in the future study.

In conclusion, we first provided the evidence that RNF146 was a cargo of XPO1-mediated nuclear export. RNF146 protected endothelial cells from Ang II-induced oxidative injury by AIF

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degradation, which was dependent on XPO1-mediated nuclear export. These results may have implications in novel treatment of endothelial cellular injury.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

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