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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

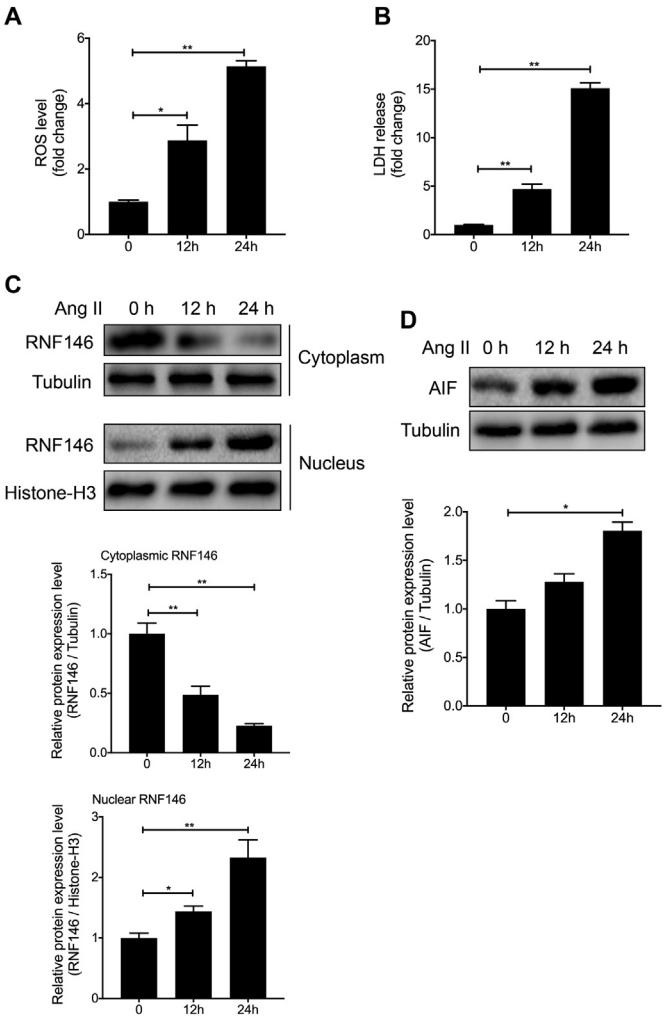

Fig. 1. Ang II induced HUVECs death and cytoplasmic-to-nuclear translocation of RNF146. (A) Intracellular ROS level assay in HUVECs after Ang II (200 nM) treatment for 12 h and 24 h. (B) Measurement of LDH release level from HUVECs after Ang II treatment for 12 h and 24 h. (C) Western blot analysis of 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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