Apocynin protects endothelial cells from endoplasmic reticulum stress-induced apoptosis via IRE1α engagement

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Abstract
Endoplasmic reticulum (ER) stress-induced endothelial cell (EC) apoptosis has been implicated in a variety of human diseases. In addition to being regarded as an NADPH oxidase (NOX) inhibitor, apocynin (APO) exhibits an anti-apoptotic effect in various cells. The present study aimed to identify the protective role of apocynin in ER stress-mediated EC apoptosis and the underlying mechanisms. We found that ER stress resulted in a significant increase in c-Jun N-terminal kinase phosphorylation, and elicited caspase 3 cleavage and apoptosis. However, apocynin obviously attenuated EC apoptosis and this effect was partly dependent on ER stress sensor inositol-requiring enzyme 1α (IRE1α). Importantly, apocynin upregulated IRE1α expression in both protein and mRNA levels and promoted the pro-survival XBP1 splicing. Our results suggest that apocynin protects ECs against ER stress-induced apoptosis via IRE1α involvement. These findings may provide a novel mechanistic explanation for the anti-apoptotic effect of apocynin in ER stress.

Keywords Apocynin · IRE1α · Endoplasmic reticulum stress · Apoptosis · Endothelial cell

Introduction
The endoplasmic reticulum (ER) functions as a dynamic organelle responsible for folding of secretory and membrane proteins. Perturbations of ER homeostasis lead to accumulation of unfolded or misfolded proteins, thus triggering ER stress. To restore the ER homeostasis, adaptive signaling cascade known as unfolded protein response (UPR) is activated. Among the three UPR branches, inositol-requiring enzyme 1α (IRE1α) is the most sensitive arm. As we have reviewed previously [1], IRE1α functions as an intriguing cell fate switch. IRE1α contains a kinase domain and an endoribonuclease (RNase) domain. The best characterized substrate for the RNase domain of IRE1α is X-box binding protein 1 (XBP1). Upon ER stress, XBP1 is spliced by IRE1α, thereby generating XBP1s, an active transcription factor encoding various adaptive genes. The IRE1α-XBP1 axis exerts adaptive and pro-survival effect, while other signaling pathways such as IRE1α-TRAF2-JNK axis evoke pro-apoptotic effect.

Endothelial cells (ECs) line the intima of blood vessels and sense the mechanical and chemical stimulations in blood flow. Influenced by the elevation or reduction of blood nutrients, ECs are very metabolically active cells. With a high amount of protein synthesis, ECs are predisposed to ER stress [2]. Stressors such as hyperglycemia and hyperlipidemia may cause ER stress. In response, the ECs initiate UPR to promote survival. However, in the case of unresolved and sustained ER stress, the UPR can result in EC apoptosis [3, 4]. Accumulating evidence showed that ER stress-induced EC apoptosis is implicated in a variety of human diseases, including diabetes [5], atherosclerosis [6], and neurodegeneration [7]. Nevertheless, the molecular mechanisms of ER stress-elicited EC apoptosis remain elusive. Thus, it is important to clarify the mechanisms and find potential therapeutic agents for these diseases.
Apocynin (APO), isolated from Picrorhiza kurroa, is widely used as an inhibitor of NADPH oxidase (NOX) [8]. In addition, mounting evidence showed the anti-inflammatory activity of apocynin in various cells and animal models of inflammation [9, 10]. Moreover, the anti-apoptotic effect of apocynin was demonstrated in recent studies as well. The protective role of apocynin in EC apoptosis was verified in sepsis [11], retinopathy of prematurity [12], and pulmonary hypertension [13]. Furthermore, our previous study revealed the protective effect of apocynin on heat stress-induced EC apoptosis [14]. Until now, most of the studies have attributed the protective effect of apocynin to the inhibition of NOX activity and reactive oxygen species (ROS) production. However, it is noted that apocynin attenuated the increased ER stress in the remote non-infarcted myocardium after myocardial infarction in rabbits [15]. Therefore, we wonder whether apocynin protects ECs from apoptosis via the regulation of ER stress signaling.

A recent study showed that IRE1α overexpression inhibited ER stress-mediated apoptosis in BMP2-stimulated ATDC5 cells [16]. Moreover, the protective effect of IRE1α in ER stress-evoked apoptosis was also described in coronavirus infection [17]. Accordingly, we hypothesized that apocynin may be involved in the regulation of ER stress sensor IRE1α expression, thereby promoting EC survival during ER stress.

In the present study, we strive to determine whether apocynin mediates the anti-apoptotic effect in ECs under ER stress via the regulation of IRE1α signaling pathways.

Materials and methods

Reagents

Apocynin (A10809) and thapsigargin (T9033) were purchased from Sigma (St. Louis, USA). The sequences of oligonucleotide for control and IRE1α siRNA were synthesized by GenePharma (Shanghai, China). The siRNA-targeted sequences were as follows: control siRNA, sense 5′-UUCUCCGAACGUGACACGTU-3′ and antisense 5′-AUCGUGACACGUUCGGAATA-3′; IRE1α siRNA, sense 5′-AGACAGAGGGCAAGACGAA-3′ and antisense 5′-UUCUCUCUUUGGCCUCUGCU-3′. Antibodies against IRE1α (#3294, CST, MA, USA), p-JNK (#4668, CST, MA, USA), JNK (#9252, CST, MA, USA), XBPl (sc-7160, Santa, CA, USA), XBPls (#647501, Biolegend, San Diego, USA), and cleaved caspase 3 (#9665, CST, MA, USA) were used. Anti-β-actin monoclonal antibody and horseradish peroxidase-conjugated species-specific secondary antibodies were from Zhongshan Golden Bridge Bio-technology (Beijing, China). TRizol reagent and the Transcriptor First Strand cDNA Synthesis Kit were from Life Technologies (Carlsbad, CA). THUNDERBIRD SYBR qPCR Mix was from Toyobo (Osaka, Japan). Annexin V-FITC/PI apoptosis detection kit was from Beyotime (Beijing, China). Other chemicals and reagents were purchased from Sigma unless indicated.

Cell culture

Human umbilical vein endothelial cells (HUVECs) were obtained from ScienCell Research Laboratories (Carlsbad, CA). Cells were maintained in Dulbecco’s modified Eagle’s medium-Ham’s Nutrient Mixture F-12 (DMEM/F12) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin (GIBCO, Grand Island, NY) at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air.

Western blotting

HUVECs were harvested and lysed in cell lysis buffer according to manufacturer’s instructions. Total protein concentrations were determined by the BCA assay. Western blotting was performed as previously described [18]. Primary antibodies against IRE1α (1:1000), p-JNK (1:1000), JNK (1:1000), XBPl (1:200), XBPls (1:500), and cleaved caspase 3 (1:1000) were used. The secondary antibody were diluted at 1:8000. Protein bands were visualized with chemiluminescence and imaged with an imaging station. Image J was used to measure the density of the bands.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using a TRizol RNA isolation system according to the manufacturer’s instructions. Complementary DNA (cDNA) was synthesized with the Transcriptor First Strand cDNA Synthesis Kit according to the manufacturer’s instructions. Real-time PCR was performed using the ABI PRISM® 7500 Sequence Detection System with a THUNDERBIRD SYBR qPCR Mix kit. The following primers were used: human IRE1α forward, 5′-GGCTTG GTTACACAAATTAGA-3′; human IRE1α reverse, 5′-TTT GGAAGCCCTTGTCTCCT-3′; human XBPl forward, 5′-CTGAGTCCGAATCGTGAGCAG-3′; human XBPls reverse, 5′-ATCCATGGGAGATGTCTTG-3′; human β-actin forward, 5′-CATGTACGTTGCTATCCAGCCG-3′; human β-actin reverse, 5′-TCCTAAATGTCACGCACGAT-3′; human GAPDH forward, 5′-TGTTCAGACGTACGCGC-3′; human GAPDH reverse, 5′-GGTGCTCTGAGCGATGTC-3′.
Annexin V-FITC/PI staining

To visually identify EC apoptosis, double staining with FITC-conjugated Annexin V and propidium iodide (PI) was conducted. After experimental treatments, HUVECs were washed twice with PBS and stained for 15 min in the dark with 5 mL Annexin V-FITC and 10 mL PI in binding buffer at room temperature. The staining results were imaged using a Zeiss LSM780 laser confocal scanning microscope (Zeiss, Oberkochen, Germany).

Statistical analysis

Data were expressed as means ± s.d. of at least three independent experiments and analyzed using SPSS version 16.0 software. Statistical comparisons were performed using a one-way analysis of variance followed by post hoc pairwise comparisons. P < 0.05 was considered significant.

Results

Apocynin attenuates ER stress-induced EC apoptosis

First, we wondered whether apocynin can alleviate ER stress-induced EC apoptosis. In order to induce ER stress in ECs, we applied thapsigargin (TG), a specific irreversible inhibitor of ER calcium-ATPase, for the following experiments. Given that JNK signaling pathway participates in ER stress-induced cell apoptosis [17], we detected the phosphorylation levels of JNK. As revealed in Fig. 1a, TG application caused a significant increase in JNK phosphorylation, while apocynin pretreatment at the concentrations ranging from 0.1 to 1 mmol/L strongly mitigated this effect. This result reveals that apocynin may protect EC from ER stress-induced apoptosis. Then, to further investigate the anti-apoptotic role of apocynin, we evaluated the cleavage of caspase 3. We found that TG evoked a significant increase in cleaved caspase 3 expression (Fig. 1b). Nevertheless, apocynin pretreatment diminished this effect robustly (Fig. 1b). Subsequently, annexin V-FITC/PI staining was conducted to verify the anti-apoptotic role of apocynin. Consistent with the western blot results, TG induced an obvious increase in EC apoptosis, while apocynin significantly alleviated ER stress-induced apoptosis (Fig. 1c, d). Hence, these data clearly highlight the anti-apoptotic role of apocynin in ER stress-mediated EC apoptosis.

IRE1α knockdown eliminates the anti-apoptotic effect of apocynin in ECs

Next, to investigate whether IRE1α is implicated in apocynin-mediated anti-apoptotic effect, we adopted the siRNA-mediated IRE1α knockdown strategy. Results showed that transfection of IRE1α siRNA significantly reduced IRE1α protein expression, as well as abolished apocynin-induced upregulation of IRE1α expression (Fig. 2a). To further clarify the role of IRE1α in APO-induced anti-apoptotic effect, expression levels of cleaved caspase 3 were detected after IRE1α siRNA treatment. The results demonstrated that IRE1α siRNA transfection augmented TG-induced caspase 3 cleavage and abolished apocynin-mitigated caspase 3 cleavage during ER stress, while control siRNA treatment had neither effect on TG-induced increase in cleaved caspase 3, nor on apocynin-mitigated caspase 3 cleavage (Fig. 2b). To fully confirm these results, we performed the annexin V-FITC/PI staining. Consistently, IRE1α knockdown abolished the anti-apoptotic effect of apocynin in ECs during ER stress. As revealed in Fig. 2c, d, in comparison to Control siRNA+APO+TG group, the IRE1α siRNA+APO+TG group showed an increased level of apoptosis significantly. Taken together, these results indicate that apocynin prevents ECs from ER stress-elicited apoptosis, and that this effect is at least partly dependent on IRE1α.

Effects of apocynin on the regulation of IRE1α expression in ECs

To determine whether apocynin mediates anti-apoptotic effect through regulation of IRE1α, we next proceeded to examine the protein expression levels of IRE1α. Total proteins were extracted from HUVECs after apocynin (0.5 mmol/L) treatment for 0, 4, 8, 12, 16, and 24 h, respectively. Then, protein expression levels of IRE1α were determined by western blotting. As shown in Fig. 3a, IRE1α protein expression was gradually increased, reached a peak at 8 h and then slightly decreased from 16 to 24 h. Next, IRE1α protein expression was examined in HUVECs subjected to different concentrations of apocynin for 8 h (Fig. 3b). The results showed that IRE1α protein expression was significantly enhanced by apocynin at the concentrations ranging from 0.25 to 1 mmol/L. To further validate the effects of apocynin on IRE1α expression, qRT-PCR assays were performed. As a result, the mRNA expression of IRE1α was obviously increased after the application of apocynin (0.5 mmol/L) for 8 h (Fig. 3c). Hence, these results suggest that apocynin upregulates the expression of IRE1α in both protein and mRNA levels.

Effects of apocynin on IRE1α-mediated XBP1 splicing during ER stress

It has been known that XBP1 was the main target of IRE1α [1]. Thus, we next detected the protein and mRNA expression levels of XBP1s in HUVECs after apocynin stimulation. Unexpectedly, apocynin exhibited no significant influence on
the expression of XBP1s, neither in protein nor in mRNA levels (Fig. 4a–c). However, when ER stress occurred, apocynin contributed to the generation of XBP1s greatly. As revealed in Fig. 4d, TG elicited a moderate degree of XBP1 splicing in HUVECs, while the application of 0.5 mmol/L apocynin in HUVECs resulted in more XBP1s production. In addition, the APO+TG group showed a significant reduction in XBP1u (the unspliced form of XBP1), compared with the TG group (Fig. 5). These results suggest that apocynin intensifies the pro-survival IRE1α-mediated XBP1 splicing, which may serve as a protective mechanism against EC apoptosis.

Discussion

ER stress-induced EC apoptosis has been implicated in a variety of pathological processes. The ER stress sensor IRE1α has long been recognized as an important cell fate switch. Apocynin, widely used as an NOX inhibitor, was also demonstrated to suppress ER stress in kidney dysfunction [19], Alzheimer disease (AD), and myocardial infarction [15]. The protective role of apocynin in ER stress was generally considered as a result of inhibition of NOX-derived ROS. However, we wondered whether...
apocynin influences IRE1α signaling pathways and thus participates in the regulation of EC apoptosis. In the present study, we found that apocynin exhibited anti-apoptotic effect in ECs during ER stress. JNK phosphorylation levels, cleaved caspase 3 expression levels, and annexin V-FITC/PI staining were examined. As expected, apocynin abated TG-induced JNK phosphorylation, caspase 3 cleavage and apoptosis in ECs. However, IRE1α knockdown significantly diminished the anti-apoptotic effect of apocynin in ECs, which implied the possibility that apocynin protects from ER stress-induced EC apoptosis via IRE1α enhancement.

Next, to the best of our knowledge, we identified the role of apocynin in the regulation of IRE1α expression for the first time. Our data showed that apocynin enhanced IRE1α expression in both protein and mRNA levels in ECs. These results suggested that IRE1α is involved in apocynin-mediated anti-apoptotic signals. Previously, we have reviewed that IRE1α-evoked XBP1 splicing promotes cell survival [1]. Therefore, we then detected the protein and mRNA expression levels of XBP1s. Interestingly, no significant alteration of XBP1s expression was observed, neither in protein nor in mRNA levels. As we all know, TG induces ER stress and activates IRE1α, which further
mediates pro-survival XBP1 splicing at the initial stage. However, if the adaptive UPR signaling pathways fail to rebalance ER homeostasis, IRE1α may promote several pro-apoptotic signaling pathways, including regulated IRE1α-dependent decay (RIDD) and IRE1α-TRAF2 axis [1]. In the present study, we found that the degree of XBP1 splicing, characterized by an increase in XBP1s and a decrease in XBP1u, was higher in TG-stimulated ECs with apocynin pretreatment when compared with TG stimulation alone. This result can be explained that apocynin enhances IRE1α expression and thus promotes the adaptability of ECs. Therefore, when ER stress occurs, XBP1 splicing process can be intensified in condition of the high-level IRE1α deposit, tilting the balance of IRE1α signaling toward EC survival. Taken together, we believe that apocynin promotes pro-survival IRE1α-XBP1 axis and thus protects ECs from ER stress-elicited apoptosis.

Until now, most studies have attributed the anti-apoptotic effect of apocynin to the inhibition of NOX. Our results provide a novel mechanism explanation for the protective role of apocynin in ER stress-induced EC apoptosis. Crosstalk between ER stress and oxidative stress was evidenced in various disease processes such as cardiovascular pathology [20]. ER stress, characterized by protein misfolding, can induce ROS production and oxidative stress [21]. Likewise, oxidative stress disturbs redox homeostasis in the ER and causes ER stress. ER stress and oxidative stress can accentuate each other and activate pro-apoptosis signaling [22]. Intriguingly, additional evidence indicate that ER stress in ECs can be uncoupled from oxidative stress and that some antioxidants can alleviate oxidative stress but not ER stress [23]. Previous study showed that the activation of NOX in myocardium mediated increased ER stress, contributing to myocyte apoptosis [15]. And siRNA knockdown of the
cytosolic subunit of NOX, p47phox, abrogated TG-induced apoptosis in H9C2 cells [24]. Using p47phox gene knockout mice, Maria found that NOX acts as an intermediate for ER stress in endothelial dysfunction [25]. Therefore, whether NOX is involved in the anti-apoptotic role of apocynin in ECs during ER stress remains to be identified.

Besides the research findings, there are also some limitations in this study. For example, the exact mechanism by which apocynin upregulates the expression of IRE1α is still yet to be explored in the future. It also remains to be further investigated on whether and how apocynin affects dimerization and autotransphosphorylation of IRE1α. IRE1α exists as a monomer in basal conditions, while the luminal domain of IRE1α undergoes dimerization upon activation. The dimerization promotes autotransphosphorylation and leads to the activation of RNase domain which is responsible for XBP1s generation. Accordingly, in the same qRT-PCR assay, XBP1s mRNA levels were normalized by corresponding GAPDH mRNA levels. HUVECs were stimulated with TG (1 µmol/L) for 8 h, with or without APO (0.5 mmol/L) pretreatment. Cell lysates were immunoblotted with anti-XBP1s antibody. The ratio of immunointensity between XBP1s and GAPDH was calculated. The results are expressed as mean ± s.d. from three independent experiments. *P < 0.05 vs. control. *P < 0.05 vs. TG alone.

Fig. 4 Effects of APO on XBP1s expression. a HUVECs were treated with APO (0.5 mmol/L) for 0, 4, 8, 12, 16, and 24 h, respectively. b HUVECs were treated with APO at the indicated dose for 8 h. For a and b, DMSO was used as solvent control. Cell lysates were immunoblotted with anti-XBP1s antibody. The ratio of immunointensity between XBP1s and GAPDH was calculated. c HUVECs were treated or untreated with APO (0.5 mmol/L) for 8 h. DMSO was used as solvent control. XBP1s and GAPDH mRNAs were run simultaneously in the same qRT-PCR assay. XBP1s mRNA levels were normalized by corresponding GAPDH mRNA levels. d HUVECs were stimulated with TG (1 µmol/L) for 8 h, with or without APO (0.5 mmol/L) pretreatment. Cell lysates were immunoblotted with anti-XBP1s antibody. The ratio of immunointensity between XBP1s and GAPDH was calculated. The results are expressed as mean ± s.d. from three independent experiments. *P < 0.05 vs. control. *P < 0.05 vs. TG alone.

In conclusion, the present study revealed that apocynin protects ECs from ER stress-induced apoptosis and this effect is partly dependent on IRE1α. Apocynin upregulates the expression of IRE1α and promotes IRE1α-mediated XBP1 splicing. Our findings shed light on the novel protective mechanism of apocynin, which may provide a
potential pharmacologic target for ER stress-associated human diseases.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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