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Pterostilbene exerts an anti-inflammatory effect via regulating endoplasmic reticulum stress in endothelial cells

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ABSTRACT

Pterostilbene (PT), an analog of resveratrol, exerts a potent anti-inflammatory effect. However, the protective effects of PT against inflammation in endothelial cells have not been elucidated. Previous studies have confirmed that endoplasmic reticulum stress (ERS) plays an important role in regulating the pathological process of endothelial cell inflammation. In this study, we explored the effect of PT on the tumor necrosis factor-α (TNF-α)-induced inflammatory response in human umbilical vein endothelial cells (HUVECs) and elaborated the role of ERS in this process. TNF-α treatment significantly upregulated the levels of inflammation-related molecules in cell culture media, increased the adhesion of monocytes to HUVECs, and enhanced the expression of the MMP9 and ICAM proteins in HUVECs. Additionally, TNF-α potently increased ERS-related protein levels, such as GRP78 and p-eIF2α. However, PT treatment reversed the increased production of inflammatory cytokines and the adhesion of monocytes to HUVECs, as well as reduced the TNF-α-induced effects exerted by ERS-related molecules. Furthermore, thapsigargin (THA), an ERS inducer, attenuated the protective effect of PT against TNF-α-induced inflammation and ERS in HUVECs. Additionally, the downregulation of ERS signaling using siRNA targeting eIF2α and IRE1 not only inhibited ERS-related molecules but also simulated the therapeutic effects of PT on TNF-α-induced inflammation. In summary, PT treatment potently attenuates inflammation in vascular endothelial cells, which at least partly depends on the reduction of ERS.

1. Introduction

Pterostilbene (trans-3,5-dimethoxy-4-hydroxystilbene; PT), a natural phytoalexin found in blueberries, is a dimethylated analog of resveratrol [1]. Due to methoxyl substitution-induced hyperlipophilicity, PT may present higher bioactivity than resveratrol [2]. Additionally, a high dietary intake of PT does not result in toxicity in mice [3], though PT has been confirmed to be toxic to tumor cells [4]. Importantly, studies have shown that PT is distributed in a wide range of tissues in the form of its metabolite, pterostilbene-4′-sulfate, after dietary administration in rats. However, the brain is an exception, where PT exists in its intact form [5]. The presence of nonmetabolized PT in the brain may indicate that it has specific central nervous system activity. Furthermore, previous studies have indicated that PT exhibits many biological actions, such as displaying anti-inflammation, anti-oxidative stress, anti-apoptosis,

Abbreviations: ATF6, activating transcription factor 6; BSA, bovine serum albumin; CCK8, cell counting kit-8; CHOP, C/EBP homologous protein; DMSO, dimethylsulfoxide; eIF2α, eukaryotic translational initiation factor 2α; ERAD, ER-associated degradation; ER, endoplasmic reticulum; ERS, endoplasmic reticulum stress; FBS, fetal bovine serum; GRP78, glucose-regulated protein 78; HUVECs, human umbilical vein endothelial cells; ICAM1, intercellular cell adhesion molecule 1; IL-8, interleukin-8; IRE1, inositol-requiring enzyme 1; LSD, least significant difference; MCP-1, monocyte chemoattractant protein-1; MMP9, matrix metalloproteinase 9; OD, optical density; PBS, phosphate-buffered saline; p-eIF2α, phosphorylated eIF2α; PERK, PKR-like ER kinase; PT, Pterostilbene; SD, standard deviation; sICAM1, soluble adhesion molecule ICAM1; siRNA, small interfering RNA; TBST, Tris-buffered saline containing Tween-20; THA, thapsigargin; TNF-α, tumor necrosis factor-α; UPR, unfolded protein response.

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anti-ischemia and anti-hyperlipidemia effects [6–8]. Notably, while PT has been found to be capable of exerting protective effects in a wide spectrum of models of inflammatory injuries both in vivo and in vitro [6,7,9], the beneficial effect induced by PT against inflammation in endothelial cells and the molecular bases underlying this process have not been clearly elucidated.

Endoplasmic reticulum (ER) homeostasis is the basis for ER functions. However, there are numerous factors leading to functional and structural disorders of the ER, referred to as ER stress (ERS), including malnutrition, hypoxia and disturbance of calcium homeostasis and protein glycosylation. ERS often results in unfolded or misfolded proteins accumulating in the ER, consequently activating the unfolded protein response (UPR) [10]. Thus, the characteristic molecules of the three UPR pathways are used to describe the development of ERS, including PKR-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6), which are collectively referred to as ERS sensors [11]. Under normal conditions, these three molecules remain inactive due to interactions with the immunoglobulin heavy chain binding protein, also known as glucose-regulated protein 78 (GRP78). However, when there is an increase in unfolded proteins in the ER lumen, GRP78 is disaggregated from UPR-related proteins and then binds to unfolded proteins to promote protein folding; additionally, UPR signaling is activated, leading to a decrease in global protein synthesis and an increase in ER-associated degradation (ERAD), which attenuates ERS and restores normal ER function. Nevertheless, when ER dysfunction continues, excessive ERS induces apoptosis [12]. In this process, the release of GRP78 from PERK, IRE1 and ATF6 leads to their activation as well as to downstream signaling involving the α-subunit of eukaryotic translational initiation factor 2α (eIF2α), C/EBP homologous protein (CHOP), and apoptotic family members [13,14]. In summary, when ERS continues, the UPR is unable to exert its normal cellular function, resulting in a transition from a pro-survival response to a pro-apoptotic response and subsequently initiating apoptosis [13–15].

Notably, ERS may represent a major basis of inflammatory responses, and inhibition of ERS may effectively attenuate inflammatory responses in endothelial cells [16,17]. In addition, studies have shown that resveratrol, an analog of PT, exerts a biological effect on ERS signaling [18,19]. Therefore, it is reasonable to hypothesize that PT exhibits pharmacological action under inflammation and ERS. In this study, we aimed to elucidate the protective effect of PT against inflammation in endothelial cells and to explore its relationship with ERS during this process.

2. Materials and methods

2.1. Cell culture, treatments and siRNA transfection

HUVECs (ATCC, Manassas, VA, USA), which were used between passage 1 and 5, were cultured in M199 media (1 mg/ml D-glucose) from Gibco (Invitrogen, Life Technologies, Ghent, Belgium), which was supplemented with 10% fetal bovine serum (FBS) (Biochrom GmbH, Germany), 2 mM L-glutamine, 30 mg/l endothelial cell growth factor supplement (EGCS), 100 IU/ml penicillin and 100 mg/ml streptomycin at 37 °C under 5% CO_2. For stimulation experiments, endothelial cells were initially seeded at a density of 2.5×10^4 cells/cm^2 and allowed to adhere for 24 h. The HUVECs were found to be >95% pure, as judged through regular fluorescence-activated cell sorter analysis (BD FACScan, Becton Dickinson, Germany) using antiserum against von Willebrand factor. PT (Sigma–Aldrich, St. Louis, MO, USA) was dissolved in DMSO and was diluted with culture media immediately prior to the experiment; the final concentration of DMSO in the cell culture experiments was 0.1% or less. The control group was treated with an equal volume of DMSO alone. Upon their reaching 80% confluence [20], TNF-α (Sigma–Aldrich, St. Louis, MO, USA) was used to induce an inflammatory response in the HUVECs according to our previous study. The cells were pre-treated with TNF-α (1, 5, or 10 ng/mL) for 6 h. Then, the effects of TNF-α on cell viability, inflammatory cytokine activity and expression of ERS-related molecules were determined. Subsequently, the cells were treated with different concentrations of PT (0.1, 0.5, or 1 μM) for 2 h, followed by further incubation in the absence or the presence of 1 μM thapsigargin (THA, an ERS inducer, Sigma–Aldrich, St. Louis, MO, USA) or eIF2α and IRE1 siRNA (described in detail below). The cells were then treated with 10 ng/mL TNF-α (based on preliminary experiments). After the treatments were applied, the cells were harvested for further analysis.

siRNA duplexes targeting eIF2α and IRE1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). For siRNA transfection, HUVECs were plated in 6-well plates and allowed to grow until they reached 70–80% confluence. The cells were transiently transfected with either the negative control or eIF2α and IRE1 siRNA at a concentration of 100 pM for 24 h using a siIMPORTER siRNA transfection kit (Qiagen Science Inc. Benelux) per the manufacturer's protocol (final siRNA concentration: 50 nM).

2.2. 3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay

The cultured HUVECs were carefully washed with PBS and then detached using 0.25% trypsin. The cells were subsequently collected via centrifugation at 800 rpm for 5 min and seeded in 96-well plates at a density of 0.6×10^4 cells per well in 100 μL of M199 media supplemented with 10% FBS for 12 h; six parallel replicates were prepared. After different treatments, 10 μL of MTT (final concentration 0.5 mg/mL), obtained from Sigma–Aldrich (St. Louis, MO, USA), was added to each well. Next, the plates were incubated at 37 °C in a 5% CO_2 humidified incubator for 4 h. MTT was converted from a yellowish solution to water-insoluble MTT-formazan, which is dark blue in color, by mitochondrial dehydrogenases in the living cells. The blue crystals were solubilized with 100 μl of DMSO, and the intensity was measured colorimetrically at a wavelength of 490 nm using a microplate reader (SpectraMax 190, Molecular Device, USA). Finally, cell viability was expressed as an OD value. All experiments were repeated three times.

2.3. Analysis of monocyte adhesion

An adhesion assay was performed using the Rose Bengal staining method [21]. This method was chosen based on its favorable comparison with the isotopic method and its ability to reduce the possibility of monocyte activation, as it eliminates the need to pre-label monocytes [22]. Briefly, HUVECs were cultured at a density of 5.0×10^4 cells in a collagen-precoated 24-well plate containing endothelial cell growth media (PromoCell GmbH, Heidelberg, Germany). After 24 h, the endothelial cells were subjected to various treatments, as noted previously. Then, the plate was washed twice with PBS to remove the stimulants, and U937 monocytes (2×10^5 cells/mL, ATCC, Manassas, VA, USA) were seeded onto confluent endothelial cells and incubated for 1 h in serum-free M199 media at 37 °C to allow adhesion. Each set of treatment conditions was performed in duplicate. Following stringent washing with PBS, non-adherent cells were removed. After completely aspirating the wash solution, 100 μL of a 0.25% solution of Rose Bengal stain in PBS was added to each well for 10 min at 25 °C.