Pterostilbene, a natural small-molecular compound, promotes cytoprotective macroautophagy in vascular endothelial cells

Lu Zhang\(^a,\)∗, LiuQing Cui\(^a\), GuangZhou Zhou\(^a\), Hongluan Jing\(^a\), YuQi Guo\(^b\), WenKai Sun\(^a\)

\(^a\) College of Bioengineering, Henan University of Technology, Lianhua Street, Zhengzhou 450001, China
\(^b\) Department of Obstetrics and Gynecology, The Third Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, China

Received 20 November 2011; accepted 5 June 2012

Abstract

Chemical modulators of macroautophagy (herein referred to as autophagy) have aroused widespread interest among biologists and clinical physicians because of their potential for disease therapy. Pterostilbene (PT), a natural small-molecular compound, has been demonstrated to inhibit oxidized low-density lipoprotein (oxLDL)-induced apoptosis in vascular endothelial cells (VECs). The aim of the present study was to investigate whether and how PT could induce VEC autophagy. PT at 0.5 or 1 μM could effectively induce autophagosome formation in human umbilical vein VECs (HUVECs). PT promoted autophagy via a rapid elevation in intracellular calcium ([Ca\(^{2+}\)]\(_i\)) concentration and subsequent AMP-activated protein kinase α1 subunit (AMPK\(\alpha_1\)) activation, which in turn inhibited mammalian target of rapamycin, a potent inhibitor of autophagy. PT-induced AMPK\(\alpha_1\) activation and autophagy were refractory to the depletion of serine/threonine kinase 11 but depended on calcium/calmodulin-dependent protein kinase kinase-β activation. Interestingly, PT stimulated cytoprotective autophagy so as to aid in the removal of accumulated toxic oxLDL and inhibit apoptosis in HUVECs. Our study provides a potent small molecule enhancer of autophagy and a novel useful tool in exploring the molecular mechanisms for crosstalk between apoptosis and autophagy. PT could serve as a potential lead compound for developing a class of autophagy regulator as autophagy-related diseases therapy.

© 2013 Elsevier Inc. All rights reserved.

Keywords: CaMKK\(\beta\); Macroautophagy; mTOR; Pterostilbene; Vascular endothelial cell

1. Introduction

Autophagy is an evolutionarily conserved, tightly regulated catabolic process in eukaryotes by which cytoplasmic cargo sequestered inside double-membrane vesicles are delivered to the lysosome for degradation [1]. Originally, autophagy was described as a self-digestion process which supplies nutrients for vital cellular functions against starvation [2]. Later, autophagy was reported to play important roles in various cellular responses including early embryogenesis [3], neural development [4] and carcinogenesis [5]. Extensive autophagy has generally been considered to result in a particular mode of cell death called autophagic cell death or second type of programmed cell death [6]. However, it has been suggested that autophagy induced under pathological conditions provides an adaptive strategy, allowing the cell to survive bioenergetic stress [7]. Thus, enhancement of autophagy may bring potential benefits to clinical therapy.

Vascular endothelial cells (VECs) line the interior surface of blood vessels and participate in many aspects of vascular biology and maintain vascular homeostasis [8]. VECs regulate a variety of functions in the cardiovascular system such as vasoconstriction and vasodilation, inflammation, angiogenesis, atherosclerosis and thrombosis [9]. In the past years, apoptosis was considered to be one of the central mechanisms leading to endothelial dysfunction and induces atherosclerotic lesion rupture [10]. The events involved in apoptosis are well understood. To date, a growing body of evidence indicates that autophagy plays vital roles in vascular pathophysiological processes, including atherosclerosis [11,12]. However, despite the increasing interest in autophagy, the process remains an overlooked phenomenon in VECs. The molecular mechanisms of endothelial autophagy are far from being completely elucidated.

Pterostilbene (PT) is a naturally occurring analogue of resveratrol found in blueberries and several varieties of grapes [13]. It possesses various pharmacologic activities, including anti-cancer, anti-inflammatory, anti-oxidant and anti-diabetes activities [14]. Although the structure of PT and resveratrol is similar [15], PT has better oral adsorption and metabolic stability because it has only one hydroxyl group. The dimethylether structure of PT enhances its lipophilicity and thus increases its membrane permeability, which leads to improved pharmacokinetic profiles over resveratrol [16]. Recently, we have demonstrated that PT can inhibit oxidized low-density lipoprotein (oxLDL)-induced VEC apoptosis [17]. However, nothing is known regarding its effect on autophagy induction in VECs. If PT could induce endothelial autophagy, what is the physiological relevance of these findings? We aimed to evaluate whether PT could induce VEC...
autophagy and the molecular mechanism(s) of action of this small-molecular compound.

2. Methods and materials

2.1. Reagents

Methyl M199 and fetal bovine serum (FBS) were obtained from Gibco. Dimethyl sulfoxide (DMSO) was purchased from Sangon Biotech. PT, chloroquine, haldolimon A1, rapamycin, acridine orange (AO), protease inhibitor cocktail, ATP, AMP, 2-deoxy-o-glucose (2-DG), anti-myc (A1), 3-methylationdene (3-MA) and 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetaetraetetacrylic acid acetoxymethyl ester (BAPTA-AM) were from Sigma-Aldrich. STO-605 was purchased from Calbiochem. The primary antibodies of von Willebrand factor (vWF), β-actin, GAPDH, serine/threonine kinase 11 (LKB1), STE20-related adaptor protein (STRA) and calcium/calmodulin-dependent protein kinase kinase-β (CaMKKK) were purchased from Cell Signaling Technology. The secondary antibodies Alexa Fluor 488 conjugated anti-rabbit IgG and anti-mouse IgG were purchased from Zhongshan Biological Technology. The primary antibodies against LC3B, phosphor (p)-acetyl-CoA carboxylase (ACC; Ser79), ACC, p-AMP-activated protein kinase (AMPK)α, LKB1 or CaMKKβ, p-mammalian target of rapamycin (mTOR; Ser2448), mTOR, p-70-kDa ribosomal protein S6 kinase (p-p70S6K; Thr389), p70S6K, p-eukaryotic initiation factor 4E binding protein 1 (p-4EBP1; Ser65), 4EBP1 and mouse protein 2 (MO25) were purchased from Cell Signalling Technology. The secondary antibodies Alexa Fluor 488 goat-anti-rabbit IgG were purchased from Zhongshan Biological Technology. AMPKcs, LKB1 or CaMKKβ targeted siRNA, control non-targeted siRNA and normal rabbit IgG were purchased from Santa Cruz Biotechnology. Fluoro3-AM and 0.02% Pluronic F127 in basic M199 medium for 1 h at room temperature and then normally cultured for 30 min. The fluorescence of Fluoro3-AM was excited with an excitation wavelength of 488 nm and fluorescence signals were collected with emission wavelength of 543 nm by CLSM. Data were analyzed with Leica Confocal Software. The [Ca2+]i was displayed as the intensity of fluorescence relative to that of control at 0 h.

2.7. Measurement of AMP and ATP

AMP and ATP were assessed by high performance liquid chromatography (HPLC) as described previously [19]. Briefly, AMP and ATP were obtained by acid extraction from PT or 2-DG/AM-treated HUVECs. A volume of 0.05 ml HClO4 (acid extract) and immediately mixed and centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was neutralized by adding a 75% volume of 1 M boricate buffer at pH 7.1 containing 4 mM EDTA. The mobile phase consisted of two eluents: 0.1 M KH2PO4 solution at pH 6.0 (Buffer A) and a 0.1 M KH2PO4 solution at pH 6.0, containing 10% (v/v) of CH3OH (Buffer B). All buffer solutions, after preparation and pH adjustment, were filtered through a 0.22 μm filter (Millipore). The chromatographic conditions were as follows: 9 min at 100% of Buffer A, 6 min at up to 23% of Buffer B, 2.5 min at up to 90% of Buffer B, 2 min at up to 100% of Buffer B, and held for 6 min. The initial condition was then restored and held for 9.5 min before the next injection. The column was monitored by absorption at 254 nm, and the flow rate was 1 ml/min. Individual standard curves for AMP and ATP were constructed with the use of known concentrations of standards injected versus the observed chromatographic peak areas. Retention time and peak areas were used to identify and quantify, respectively, the generated AMP and ATP.

2.8. Determination of intracellular calcium ([Ca2+]i) concentration

[Ca2+]i measurements were obtained from HUVECs preloaded with the Ca2+-sensitive fluorescent dye Fluoro3-AM by using CLSM. Briefly, cells were loaded with 10 μM Fluoro3-AM and 0.025% Pluronic F127 in basic M199 medium for 1 h, then incubated at 37°C and then normally cultured for 30 min. The fluorescence of Fluoro3-AM was excited with an excitation wavelength of 488 nm and fluorescence signals were collected with emission wavelength of 543 nm by CLSM. Data were analyzed with Leica Confocal Software. The [Ca2+]i was displayed as the intensity of fluorescence relative to that of control at 0 h.

2.9. Cell viability assay

HUVECs were plated in 96-well cell culture plates. When cells were grown to 80% confluence, they were washed once with basal M199 medium, incubated with concentrations of PT for 3, 6, 12 or 24 h. Cell viability was determined by MTT assay as described [20]. The viability (%) was expressed as (optical density (OD) of treated group/OD of control group) × 100%. The viability of the control group was set to 100%.

2.10. Preparation of oxLDL and Dil-oxLDL

Human native LDL was isolated from human plasma by sequential ultrafiltration. LDL (2mg protein/ml) was oxidized by exposure to 10mM CuSO4 for 24 h at 37°C. The extent of oxidation was determined by measuring the amount of thiobarbituric acid-reactive substances. OxLDL was labeled with the fluorescent probe, Dil and re-isolated by ultracentrifugation as described [21]. Briefly, the resultant product was exhaustively dialyzed against PBS (pH 7.4), sterilized by membrane filtration, and then aseptically packaged in a solution containing PBS at pH 7.4 and 0.2 mM EDTA.

2.11. Statistical analyses

All experiments were performed in duplicate and repeated at least 3 times. Data are expressed as mean ± S.E. Treatment groups were compared by one-way analysis of variance with SPSS 17.0 (SPSS, Chicago, IL, USA). Differences were considered statistically significant at P<0.05.

3. Results

3.1. PT induced autophagy in HUVECs

First, we detected whether PT could induce autophagy in HUVECs by AO staining. Control cells displayed green fluorescence in cytoplasm and nucleus, but cells treated with 0.5 or 1 μM of PT for 3 or 6 h showed increased red fluorescent dots in cytoplasm, indicating the formation of acidic autophagolysosomal vacuoles (Fig. 1A). To confirm the promotion role of PT on VEC autophagy, we detected the distribution of endogenous LC3 by immunostaining assay. As shown in Fig. 1B, incubating HUVECs with 1 μM of PT for 6 h markedly increased the numbers of LC3-positive puncta as compared with the control, which appeared in similar variation tendency to AO staining. We next investigated
the effect of PT on LC3 processing and LC3-II accumulation by western blot analysis. LC3 processing, namely increased ratio of LC3-II/β-actin, was obviously enhanced in HUVECs treated with 1 μM of PT for 3 or 6 h, indicating that PT might induce autophagy in HUVECs (Fig. 1C).

However, rather than inducing autophagy, an increase in LC3-II accumulation could instead be due to PT blocking autophagosome fusion with lysosomes and degradation of LC3-II. To determine the mechanism of PT action, we evaluated the autophagic flux in HUVECs. As a specific substrate for autophagy, p62 is used to monitor autophagic flux [22]. Treatment with 1 μM of PT for 3 or 6 h decreased p62 levels obviously. On the basis of these results, all subsequent experiments involved 1 μM of PT. In addition, we used chloroquine and bafilomycin A1, two compounds that increase lysosomal pH and prevent fusion of autophagosomes with lysosomes, to further demonstrate that PT provoked autophagosome formation. Treatment with chloroquine (3 μM) or bafilomycin A1 (100 nM) increased LC3-II accumulation. Incubating HUVECs with PT and chloroquine or bafilomycin A1 had a more remarkable accumulation of LC3-II (Fig. 1D). Together, these data clarified that PT induced autophagy through provoking autophagosome formation instead of causing a blockade of autophagosome/lysosome fusion in HUVECs and reflected an increase in functional autophagy.

3.2. AMPKα1 was required for PT-mediated autophagy

To investigate whether PT-induced autophagy was associated with AMPK activation, we measured the phosphorylation levels of AMPK by western blot analysis. After 10 min, PT treatment increased AMPK phosphorylation on threonine 172 without affecting the total AMPK levels (Fig. 2A). ACC is a well-established downstream target of AMPK and is activated by phosphorylation of Ser79. Our results showed a similar time course for ACC activation as that for AMPK (Fig. 2A). These data suggested that PT activated AMPK in HUVECs.

Next, we investigated whether AMPK activation was involved in PT-induced autophagy. Although endothelial cell expresses both α subunits, which are the critical catalytic subunits of AMPK, AMPKα1 is expressed at much higher levels than AMPKα2 [23]. In our study, we found that AMPKα1 but not AMPKα2, was expressed in cultured HUVECs (data not shown). We used knockdown of AMPKα1 by siRNA, which inhibited AMPKα1 protein expression by more than 80% after transfection for 48 h (Fig. 2B). Under these conditions, the increased LC3-II levels in PT-treated cells were almost completely inhibited (Fig. 2B). We confirmed these results by detecting the distribution of endogenous LC3. Transfection with AMPKα1-siRNA abrogated the effect of PT on increased LC3 spots (Fig. 2C). These data suggested that AMPK, more precisely AMPKα1, was required for PT-mediated autophagy in HUVECs.

3.3. PT activated AMPK to induce autophagy via downstream mTOR signaling

We further to detect whether mTOR signaling was involved in PT-induced AMPK activation and VEC autophagy. We first examined phosphorylation status of mTOR and its two targets, p70S6K and 4EBP1 after PT treatment. As shown in Fig. 3A and B, PT at 1 μM attenuated phosphorylation of mTOR, p70S6K and 4EBP1, indicating...
that mTOR signaling was involved in PT-induced VEC autophagy. In addition, transfection of HUVECs with AMPKα1-siRNA, but not control siRNA, blocked PT-induced mTOR signaling inhibition (Fig. 3A and B). Rapamycin, an inhibitor of mTOR signaling, that has been demonstrated to significantly promote VEC autophagy, did not affect AMPK phosphorylation (Fig. 3C and D). We concluded that PT induced autophagy by AMPK activation and the downstream inhibition of mTOR signaling pathway.

3.4. CaMKKβ, rather than LKB1, mediated PT-induced AMPK activation and VEC autophagy

In VECs, the phosphorylation of AMPK at Thr 172 is primarily controlled by two major AMPK kinases, LKB1 and CaMKKβ [24]. We next investigated whether these AMPK kinases were responsible for PT-induced AMPK activation and autophagy. LKB1 activation is tightly controlled by ATP levels via changes in the AMP/ATP ratio [25]. We
examined whether PT affected ATP levels. We found no effect of PT (1 μM) on AMP/ATP ratio in HUVECs at different time points after treatment (Fig. 4A). However, PT did not increase these proteins levels in HUVECs after treatment for 3 or 6 h (Fig. 4B). Furthermore, treatment with LKB1 siRNA did not influence PT-induced AMPK activation (Fig. 4C) and autophagy (Fig. 4D and E), indicating that LKB1 activation was not required for PT-induced AMPK phosphorylation. AMPK also could be activated by CaMKKβ in response to an increase in [Ca2+]i. We next detected whether PT modulated [Ca2+]i, in HUVECs. PT stimulated a rapid and transient increase in [Ca2+]i, followed by a decrease to the level of control cells (Fig. 5A and B). BAPTA-AM, a chelator of [Ca2+]i, prevented PT-induced AMPK activation and LC3-II accumulation (Fig. 5C-E). These results showed that [Ca2+]i played an essential role in PT-induced phosphorylation and activation of AMPK. Furthermore, we found that STO-609, a CaMKKβ inhibitor, effectively reduced the effects of PT on phosphorylation of AMPK and ACC and completely abrogated LC3-II accumulation (Fig. 6A-C). Treatment with CaMKKβ siRNA effectively inhibited PT-induced AMPK activation and autophagosome formation (Fig. 6D-F). Taken together, our data suggested that Ca2+/CaMKKβ, rather than ATP/LKB1, mediated PT-induced AMPK activation and autophagy.

3.5. PT reduced the accumulation of oxLDL and apoptosis by enhancing autophagy in HUVECs

We sought to assess the functional relevance of our findings. Previously, we found that PT could protect against oxLDL-induced HUVEC apoptosis [17]. OxLDL also induced autophagy, which plays a cytoprotective role in removing abnormal proteins and oxLDL accumulation [27]. Thus, we speculated that one possible mechanism...
underlying the anti-apoptotic effect of PT may be through inducing autophagy, which in turn reduced oxLDL accumulation and apoptosis in HUVECs. We found that treatment with PT (0.1–1 μM) alone did not affect HUVEC viability (Fig. 7A), suggesting that PT-induced autophagy did not lead to cell death.

Next, we detected whether PT could enhance oxLDL-induced autophagy. Incubating HUVECs with oxLDL induced cell autophagy as previously reported [27]. Unexpectedly, the increase in LC3-II levels in HUVECs cotreated with oxLDL and PT were no different than those of HUVEC exposed to oxLDL or PT alone (Fig. 7B). It is known that LC3-II can be rapidly degraded after autophagosome fusion with lysosomes. Disrupting lysosomal function by bafilomycin A1, cells treated with PT and oxLDL had higher levels of LC3-II than cells treated with oxLDL alone (Fig. 7B). In addition, p62 levels in HUVECs treated with oxLDL plus PT were significantly lower than those of cells treated with oxLDL alone (Fig. 7B), indicating that PT promoted fusion of autophagosomes with lysosomes, a definitive event in the induction of cellular autophagy, in oxLDL-treated HUVECs. To further examine whether PT-induced autophagy reduced the accumulation of oxLDL, we treated HUVECs with Dil-oxLDL. The results showed that the accumulation of oxLDL in HUVECs treated with PT or rapamycin decreased (Fig. 7C–E).

Phase-contrast microscopy was performed to evaluate whether PT-involved defense against oxLDL-induced apoptosis was, at least partially, due to PT-induced autophagy. Cotreatment with ATG5 siRNA, oxLDL and PT, the number of shrinking cells and cells detached from the culture dish were obviously increased in comparison with control siRNA, oxLDL and PT cotreated-group (Fig. 7F). Parallel experiments with TUNEL staining again showed an increase in apoptosis of HUVECs treated with oxLDL and PT when the ATG5 levels were down-regulated by ATG5 siRNA (Fig. 7G). Together, these results demonstrated that PT stimulated cytoprotective autophagy so as to aid in the removal of accumulated toxic oxLDL and inhibit apoptosis in HUVECs.

4. Discussion

Accumulating data have demonstrated the benefits of resveratrol in preventing cardiovascular diseases [28]. Unfortunately, the cardiovascular protective effect of resveratrol is limited by its low bioavailability. PT, a structurally related and naturally occurring small compound, shows longer half-life and higher bioavailability in vivo [16]. PT was shown to have an inhibitory effect on the growth of human vascular smooth muscle cells [29], suggesting that this small-molecular compound may be a potential candidate for treating vascular diseases such as atherosclerosis. However, the possible mechanism by which PT can affect the vascular diseases remains unclear. VEC apoptosis, mainly induced by oxLDL, is commonly considered as a pivotal role in atherosclerosis both in the early stages

Fig. 5. PT regulated the [Ca2+]i. (A) Immunofluorescent photographs reflected the relative levels of [Ca2+]i in HUVECs treated with or without 1 μM of PT for 0–360 min. Bar=20 μm. (B) The line graph indicated the relative levels of [Ca2+]i. (C) Western blot analysis of LC3-II accumulation and the phosphorylation of AMPK in HUVECs treated with PT (1 μM), BAPTA-AM (20 μM) or PT+BAPTA-AM, respectively. (D) Bar graph showed the relative level of p-AMPK. (n=3, **P<.01 vs. control, #P<.05 vs. PT). (E) Bar graph showed the relative level of LC3-II. (n=3, *P<.05 vs. control, #P<.05 vs. PT).
of lesion formation, and later in the process of disease development by inducing atherosclerotic plaque instability [30]. Recently, we have demonstrated that PT could inhibit oxLDL-induced VEC apoptosis, indicating that PT has endothelium-protective effects [17]. However, whether PT could affect endothelial autophagy has not been studied. The role of autophagy on VEC fate decision was largely undefined. In this study, we reported for the first time that PT could induce autophagy in HUVECs. These data suggested that one major mechanism underlying the anti-apoptotic effect of PT might be through removing oxLDL accumulation in HUVECs dependent on AMPK-mediated AMPK activation and autophagy. (A) Western blot analysis of LC3-II accumulation and the phosphorylation of AMPK and ACC in HUVECs treated with STO-609 (10 or 20 μg/ml) and in the absence or presence of PT (1 μM). (B) Bar graph showed the relative levels of p-AMPK and p-ACC. (*P<0.05, **P<0.01 vs. control, #P<0.05, PT+STO-609 (10 μg/ml) vs. STO-609 (10 μg/ml), +P<0.05, PT+STO-609 (20 μg/ml) vs. STO-609 (20 μg/ml). ##P<0.01 vs. PT+control siRNA). (C) Bar graph showed the corresponding level of LC3-II. (n=3, *P<0.05 vs. control, #P<0.05, PT+STO-609 (10 μg/ml) vs. STO-609 (10 μg/ml), ++P<0.01, PT+STO-609 (20 μg/ml) vs. STO-609 (20 μg/ml)). (D) Western blot analysis of CaMKKβ and AMPK in HUVECs transfected with CaMKKβ-siRNA or control siRNA for 48 h then treated with 1 μM of PT for 3 h. Bar graph showed the relative levels of CaMKKβ, p-ACC and p-AMPK. (n=3, *P<0.05, **P<0.01 vs. control siRNA, #P<0.05 vs. PT+control siRNA, ##P<0.01 vs. PT+control siRNA). (E) Immunofluorescent photographs showed endogenous punctuate of LC3 in HUVECs transfected with CaMKKβ-siRNA or control siRNA for 48 h then treated with 1 μM of PT for 3 h. Bar=10 μm. Bar graph showed the quantification of endogenous LC3 punctuate per cell. (n=3, *P<0.05 vs. control siRNA, #P<0.05 vs. PT+control siRNA). (F) Western blot analysis of LC3-II accumulation in HUVECs transfected with CaMKKβ-siRNA or control siRNA for 48 h then treated with 1 μM of PT for 3 h. Bar graph showed the relative level of LC3-II. (n=3, *P<0.05 vs. control siRNA, #P<0.05 vs. PT+control siRNA).
evidence for a contribution by LKB1. Several studies pointed that LKB1 might play a major role in regulating the activity of AMPKα2 subunit and that other, LKB1-independent, mechanisms regulate the activity of AMPKα1 [37]. Our results showed that AMPKα1, but not AMPKα2, activated in PT-treated cells and this may explain why PT-induced AMPK activation was dependent on CaMKKβ rather than LKB1.

In summary, we investigated the effects of PT on VEC autophagy and its possible molecular mechanism. We provided the first evidence that PT promoted an increase in functional and cytoprotective autophagy via a rapid elevation in [Ca2+]i and subsequent CaMKKβ activation, which in turn activated the AMPKα1-mTOR pathway. These findings enabled us to better understand why PT could inhibit oxLDL-induced VEC apoptosis so effectively. PT could be served as a useful tool to study the molecular mechanisms for crosstalk between apoptosis and autophagy and provide a potential lead compound for developing a class of autophagy regulator as autophagy-related diseases therapy.
Acknowledgments

This work was financially supported by the National Natural Science Foundation of China (no. 31101001), the Science and Technology Developmental Project of Zhengzhou (no. 20110942), and the doctoral scientific research start-up foundation from Henan University of Technology (nos. 2011BS013 and 2011BS017).

References