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Regular paper

Induction of apoptosis through the up-regulation of endoplasmic reticulum stress sensors by 5-hydroxy-7-(4″-hydroxy-3″-methoxyphenyl)-1-phenyl-3-heptanone

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The diarylheptanoid, 5-hydroxy-7-(4″-hydroxy-3″-methoxyphenyl)-1-phenyl-3-heptanone (HPH), is isolated from rhizomes of *Alpinia officinarum*. There is no reported biological function for this compound other than the inhibition of pancreatic lipase. Cell viability, the expression of endoplasmic reticulum (ER) stress genes, the activation of ER stress sensors, and the induction of apoptosis and autophagy were confirmed following HPH treatment of PC12 cells. No cytotoxicity was observed when the cells were treated with 50 μg/ml HPH, but 40% cell death was observed using MTT assays with 100 μg/ml HPH. Although HPH did not change the expression of the ER chaperones PDI, binding BiP, and calnexin, it upregulated the expression of genes for the ER stress sensors ATF6, eIF2α, and PERK. HPH also induced apoptosis *via* the activation of ATF6 fragmentation, the phosphorylation of eIF2α, and XBP1 mRNA splicing. Eventually, the results of this study demonstrated that HPH induces apoptosis through upregulation of gene expression of ER stress sensors, which may provide a basis for the development of new drugs using HPH.

Keywords: HPH, PC12 cells, ER, ER chaperones

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Abbreviations: ATF6, activating transcription factor 6; ER, endoplasmic reticulum; HPH, 5-hydroxy-7-(4″-hydroxy-3″-methoxyphenyl)-1-phenyl-3-heptanone; IRE1, inositol requiring enzyme 1; PERK, PKR like ER kinase

INTRODUCTION

Alpinia officinalum Hance is a perennial herbaceous root of the family Zigiberaceae. It is cultured widely throughout Asia, and is used in medicine or as an edible plant (Akbar, 2020). The main chemical components include volatile oils, diarylheptanoids, and flavonoids (Basri *et al.*, 2017). Pharmacological studies have indicated that *A. officinalum* exhibits several different biological functions, including inhibition of 5α-reductase activity, vasorelaxation, antioxidant activity, the generation of reactive oxygen species (ROS), cyclooxygenase (COX)-2 suppression, anti-inflammatory effects, anti-*Helicobacter pylori* effects, and suppression of gastrointestinal diseases (Gong *et al.*, 2018; Honmore *et al.*, 2016; Kim *et al.*, 2003; Kim *et al.*, 2006; Lee *et al.*, 2003; Lee *et al.*, 2008; Lee *et al.*, 2009; Park *et al.*, 2005). It has been reported that 5-hydroxy-7-(4″-hydroxy-3″-methoxyphenyl)-1-phenyl-3-heptanone (HPH) is one of the five diarylheptanoids from the rhizomes of *A. officinarum*, and for which the chemical structure has been determined (Shin *et al.*, 2004; Liu *et al.*, 2005). However, no studies have been conducted into its function as a pancreatic lipase inhibitor (Shin *et al.*, 2004). More studies into the biological function of HPH are required.

The endoplasmic reticulum (ER) of the eukaryotic cell is a critical site for posttranslational modification, including protein folding and assembly (Schwarz & Blower, 2016). Some physical disorders that disrupt ER function lead to the accumulation of misfolded proteins in the ER lumen. ER stress induces an adaptable signal called the unfolded protein response (UPR), which acts to maintain ER homeostasis via the activation of ER chaperones. Three types of ER stress sensors are activated: inositol requiring enzyme 1 (IRE1), PKR-like ER kinase (PERK), and activating transcription factor 6 (ATF6). UPR in mammalian cells is triggered by the dissociation of BiP from stress transducers such as PERK, ATF6, and IRE1 (Lai *et al.*, 2007; Schröder & Kaufman, 2005; Zheng *et al.*, 2019).

As it is known that UPR and other intercellular signals are correlated, some natural chemicals have recently been targeted for the development of new drugs by targeting the UPR signal pathway (Peng *et al.*, 2012). In this study, we aimed to clarify whether HPH, one of the diarylheptanoids, induces the UPR in PC12 cells. We found that HPH enhanced the expression of ER stress sensors, which induced apoptosis (Song *et al.*, 2017). We also examined whether the results of the UPR affected the induction of apoptosis or autophagy. An understanding of the molecular mechanisms underlying HPH-induced UPR is valuable for the development of new natural medicinal products and health supplements.

MATERIALS AND METHODS

Cell culture and sampling of HPH

The PC12 cell line has two types of cells, neuroplastic cells and eosinophilic cells. The cells were purchased from American Type Culutre Collection (cat. no. CRL-1721; Manassas, VA, USA) and cultured in collagen-coated flasks containing 85% RPMI-1640 medium, supplemented with 25 mM HEPES buffer, 10% heat-inactivated horse serum, 5% heat-inactivated fetal bovine serum, 2 mM

L-glutamine, 1 mM sodium pyruvate, 1 g/l D-(+)-glucose, 25 μg/ml streptomycin and 25 U/ml penicillin (all supplied by Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The cells were maintained in a humidified incubator at 37°C at 5% CO_2 and the medium was changed every two days. 5-hydroxy-7-(4''-hydroxy-3''-methoxyphenyl)-1-phenyl-3-heptanone (HPH) derived from *Alpinia officinarum* Hance and purified to >95.0% using high performance liquid chromatography was provided by the National Development Institute of Korean Medicine (NIKOM: Gyeongsan-Si, Gyeongsangbuk-Do, South Korea).

MTT assay

The cells were seeded in 96-well plates (at 60 to 80% confluence) and treated with HPH at concentrations of 1, 5, 10, 50, and 100 μg/ml on cell viability using a 3-(4,5-D-imethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay from Sigma-Aldrich. MTT solution (0.5 mg/ml) was added to each well, and the plates were incubated for an additional 4 h at 37°C. Following removal of the medium, the formazan crystals were solubilized in DMSO (Sigma-Aldrich; Merck KGaA). Color development was monitored at 595 nm with a reference wavelength of 650 nm, using the Sunrise™ microplate reader (Tecan Trading AG, Männedorf, Switzerland).

RT-PCR

The expression of each gene was determined using RT-PCR as described below. RT-PCR conditions included 30 cycles as follows: 94°C for 30 sec, 58°C for 30 sec and 72°C for 1 min (10 min in the final cycle) using the primers with *Taq* DNA polymerase (Solgent Co., Ltd., Daejeon, Korea). The RT-PCR primers were supplied by Bioneer Corporation (Daejeon, Korea). All chemicals were purchased from Sigma-Aldrich; Merck KGaA. The RT-PCR primers used in this study were IRE1 (5'-ACC ACC AGT CCA TCG CCA TT-3' and 5'-CCA CCC TGG ACG GAA GTT TG-3'), Binding immunoglobulin protein (BiP) (5'-AGT GGT GGC CAC TAA TGG AG-3' and 5'-TCT TTT GTC AGG GGT CGT TC-3'), ATF6 (5'-CTA GGC CTG GAG GCC AGG TT-3' and 5'-ACC CTG GAG TAT GCG GGT TT-3'), Protein disulfide isomerase (PDI) (5'-ATC GAG TTC ACC GAG CAG AC-3' and 5'-TCA CAG CTT TCT GGT CAT CG-3'), PERK (5'-GGT CTG GTT CCT TGG TTT CA-3' and 5'-TTC GCT GGC TGT GTA ACT TG-3'), XBP1 (5'-AAA CAG AGT AGC AGC TCA GAC TGC-3' and 5'-TCC TTC TGG GTA GAC CTC TGG GAG-3'), calnexin (5'-GGG AGT CTT GTC GTG GAA TTG-3' and 5'-TGC TTT CCA AGA CGG CAG A-3'), LC3a (5'-GCC TGT CCT GGA TAA GAC CA-3' and 5'-GTT CAC CAG CAG GAA GAA GG-3'), Beclin (5'-GTG CTC CTG TGG AAT GGA AT-3' and 5'-GCT GCA CAC AGT CCA GAA AA-3'), Bcl-xl (5'-CCC CAG AAG AAA CTG AAC CA-3' and 5'-GCA GAA CTA CAC CAG CCA CA-3'), Bax (5'-AGG GGC CTT TTT GTT ACA GG-3' and 5'-GAT CAG CTC GGG CAC TTT AG-3'), Bcl2 (5'-AAG CTG CAC AGC GGG GCT A-3' and 5'-CAG ATG CCG GTT CAG GTA CT-3'), Bak (5'-TTA CCT CCA CCA GCA GGA AC-3' and 5'-ACC ACC TCT CTG TGC AAT CC-3'), and GAPDH (5'-ACA TCA AAT GGG GTG ATG CT-3' and 5'-AGG AGA CAA CCT GGT CCT CA-3').

Western blot analysis

PC12 cells were collected and lysed using RIPA buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF). To measure the total protein concentration, Pierce® BCA protein Assay Kits by Thermo Scientific (Catalogue number: 23225) were used. Proteins were separated using SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The resulting membrane was incubated with primary antibodies overnight at 4°C, followed by incubation with secondary antibodies. Blots were developed using an enhanced chemiluminescence western blotting detection system Kits (Amersham, Sweden). Anti-ATF6 antibody, anti-eIF2α & anti-eIF2α-P antibodies, and goat anti-actin antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

XBP1 mRNA splicing

To amplify the spliced and unspliced XBP1 mRNA, XBP1 mRNA was amplified using the primers described above. After *Pst*1 restriction enzyme treatment of PCR products, the results were subjected to electrophoresis on 2.5% agarose gel. The size difference between spliced and unspliced XBP1 mRNA was measured.

Hoechst 33342 staining

Following treatment with HPH, PC12 cells were incubated for 30 min with Hoechst 33342 (Molecular Probes; Thermo Fisher Scientific, Inc.) loading dye and washed three times in ice-cold 1X PBS. Following staining for 10 min, the stained cells were monitored using a fluorescence microscope (Axio Scope A1; Zeiss GmbH, Jena, Germany) at 340 nm.

Statistical analysis

For statistical significance between multiple groups a one-way analysis of variance (ANOVA) test was used. Analysis performed using GraphPad Prism 6 software (GraphPad Software Inc.). Mean ±S.E.M. n = 6. $*p<0.05$ $**p<0.005$ $***p<0.001$ $****p<0.0001$.

RESULTS AND DISCUSSIONS

Cell viability and induction of ER stress

HPH is one of the diarylheptanoids which can be purified from the root of *Alpinia officinalum*, a member of the family *Zigiberaceae*, which is widely cultivated in Asia as a food and for traditional medicines (Akbar, 2020; Basri *et al*., 2017). Its chemical formula is $C_{20}H_{24}O_4$ and its molecular molar mass is 328.4 g/mol (Shin *et al*., 2004). The chemical structure is shown in Fig. 1A. Although several pharmacological effects of *Alpinia officinalum* extract have recently been identified, the only reported physiological use of purified HPH has been as a pancreatic lipase inhibitor (Liu, 2005). In this study, a PC12 cell line was used. This cell line has been used extensively in *in vitro* studies to examine neuronal diseases of the brain. Although a 100 μg/ml concentration of HPH produced a few dead cells, no morphological changes were visible in the cells following HPH treatment at concentrations below 50 μg/ml. This finding suggested that HPH induces a small amount of cell death. The authors tested the effects of HPH on cell viability using MTT assays following HPH treatment with 1, 5, 10, 50, and 100 μg/ml for 24 h, according to the manufacturer's instructions. MTT assays showed that, although 40% cell death was observed following treatment with 100 μg/ml of HPH, no cytotoxicity was observed at 50 μg/ml HPH, so an

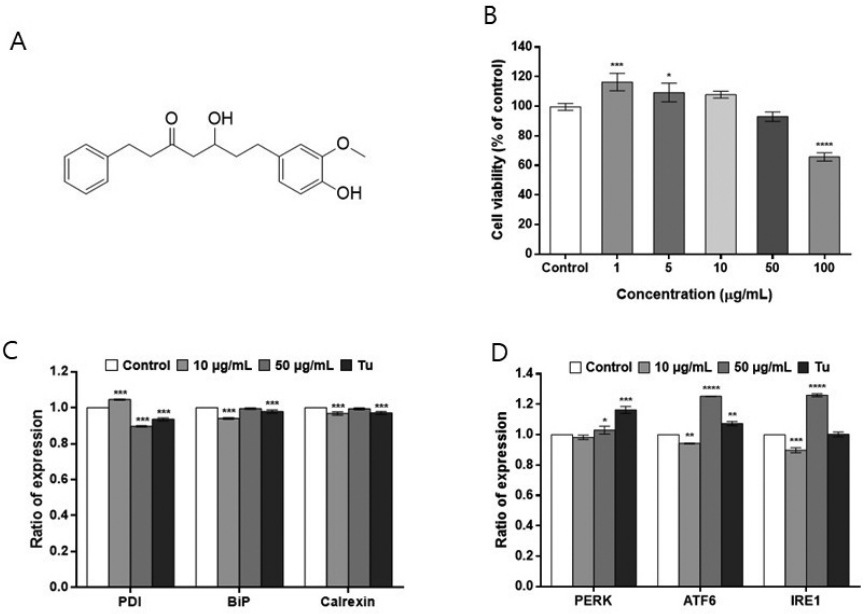

Figure 1. Effect of HPH on cell viability and expression of ER chaperone genes.
(**A**) Molecular structure of HPH (chemical formula, $C_{20}H_{24}O_4$; molar mass, 328.4 g/mol). (**B**) PC12 cells were treated with 1, 5, 10, 50 or 100 μg/ml HPH for 24 h. Cell viability was measured by MTT assay. (**C, D**) PC12 cells were treated with 10 or 50 μg/ml HPH for 24 h. Gene expression of ER chaperones and ER chaperones was demonstrated using RT-PCR. PDI, protein disulfide isomerase; BiP, binding immunoglobulin protein; PERK, protein kinase RNA-like endoplasmic reticulum kinase; ATF6, activating transcription factor-6; IRE1, inositol requiring protein 1.

HPH concentration of 50 μg/ml or less was used in the following experiments (Fig. 1B).

The main function of the intracellular organelle ER is to play a role in posttranslational modifications, including folding and assembly of cell membranes and secretory chaperone proteins (Schwarz & Blower, 2016). In the MTT assay, HPH decreased the cell viability at concentrations greater than 50 μg/ml, indicating that HPH acts as a cell stressor to some extent. The expression of ER stress chaperone genes induced by HPH treatment was tested in PC12 cells. PDI is one of the redox chaperones in the ER lumen (Wilkinson & Gilbert, 2004). One of the most abundant ER chaperones in the ER lumen is an immunoglobulin-binding protein called BiP, which is a key regulator of the ER stress response for subsequent folding and oligomerization of newly synthesized proteins (Pobre *et al.,* 2019). Calnexin is an integral protein of the ER membrane which ensures that only correctly folded glycoproteins survive (Kozlov & Gehring, 2020). As shown in Fig. 1C, the expression of the ER stress chaperones PDI, BiP, and calnexin was not changed after treatment with HPH.

Expression and activation of ER sensors

Although HPH did not induce increased cell viability and ER chaperone expression, we examined the expression of an ER stress sensor and its activation by HPH treatment of PC12 cells. When the environment of the ER becomes abnormal, the ER stress signal pathway is mediated primarily through the activation of three ER stress sensors (Lai *et al.*, 2007; Schröder & Kaufman, 2005; Zheng *et al.*, 2019). IRE1 activates XBP mRNA cleaving, generating an active XBP1 protein. PERK induces the phosphorylation of eIF2α, which inhibits translation. Active ATF6 is cleaved at its cytosolic face, and the resulting N-terminal cytoplasmic domain binds to the ER stress response element, which enhances the expression of genes for ER chaperones.

We evaluated the expression of each ER stress sensor gene under the experimental conditions described in Fig. 1C. As shown in Fig. 1D, treating PC12 cells with 50 μM HPH for 24 h did not lead to increased expression of PERK, but mRNA expression was increased by approximately 1.3 times in both ATF6 and IRE1 compared to control. This result suggests that although HPH does not directly regulate the expression of ER chaperones, it regulates the expression of genes for ER stress sensors. The activation of each ER stress sensor was investigated. As shown in Fig. 2, ATF6 fragmentation following HPH treatment was increased about 1.9 times compared with control, and the phosphorylation of eIF2α was increased 1.4 times (Fig. 2A). However, there was little change in the cleavage of XBP1 mRNA in response to ER stress (Fig. 2B). In summary, it has been shown that HPH treatment of PC 12 cells leads to an active regulation of ER stress sensors expression, rather than the regulation of the expression of ER chaperone genes.

Induction of apoptosis

Based on the results shown in Figs. 1 and 2, we concluded that HPH does not induce the expression of ER chaperones, but ER stress sensors are activated. These findings provide new insights into the effects of mild ER stress through ATF6 fragmentation and eIF2α-phosphorylation. The XBP1 mRNA splicing signaling pathway has an important role in protecting cells from damage by HPH. We investigated the role of ER stress in both apoptosis and the induction of autophagy using HPH treatment (Song *et al.*, 2017).

Figure 3A shows that HPH induces only the expression of the pro-apoptosis proteins Bax and Bak. There was no significant expression of the anti-apoptosis proteins Bcl2 and Bcl-xl or the autophagy genes LC3a and Beclin. Apoptotic bodies were observed in cells treated with HPH, using Hoechst 33342 staining (Fig. 3B).

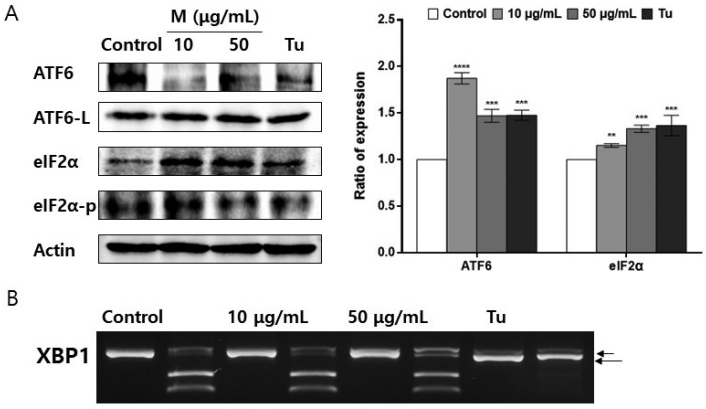

Figure 2. Effect of HPH on the activation of ER stress sensors.
(**A**) PC12 cells were treated with 10 or 50 μg/ml HPH for 24 h. ATF6 fragmentation and PERK phosphorylation were confirmed by western blotting. (**B**) HPH induces XBP1 mRNA splicing. The RT-PCR product was digested by *Pst*I to reveal a restriction site that was lost following XBP1 splicing under ER stress. The resulting XBP1 cDNA products were revealed on a 2% agarose gel. Unspliced XBP1 mRNA is indicated by a large arrow. eIF2α, eukaryotic translation-initiation factor 2α; XBP-1; X-box binding protein 1.

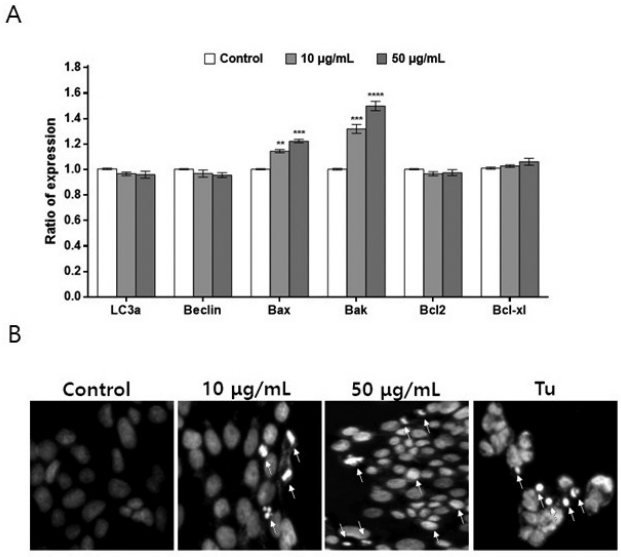

Figure 3. HPH induces apoptosis.
(**A**) Effect of HPH on expression of Bax and Bak. PC12 cells were exposed to HPH at 10 or 50 μg/ml for 24 h. Gene expression was measured using RT-PCR. (**B**) The cells were treated with 10 or 50 μg/ml HPH for 24 h, and stained with Hoechst 33342 solution to detect the formation of apoptotic bodies, as indicated by arrows. Stained nuclei were observed under a fluorescent microscope using a blue filter (×200). LC3a, microtubule-associated protein 1A/1B-light chain 3a; Bax, Bcl2 associated X; Bak, BCL2 Antagonist/Killer 1; Bcl2, B-cell lymphoma 2; Bcl-xL, B-cell lymphoma-extra larges. The same analysis as Fig. 2. was performed.

These results indicated that HPH was directly associated with the induction of apoptosis in PC12 cells. This result provides evidence to support the hypothesis that HPH induces apoptosis through ER stress signaling. However, HPH does not appear to be associated with autophagy formation.

This study demonstrated for the first time that HPH induces apoptosis via the activation of ER stress sensors mediated by ATF6 fragmentation, phosphorylation of eIF2α, and XBP1 mRNA splicing, not via the induction of the ER chaperones PDI, BiP, and calnexin. Purified HPH from *A. officinalum* extract induced apoptosis via ER stress sensor activation. This finding could provide new insights into the development of drugs and food additives.

Conflict of interest

None of the authors of this study has any financial interest or conflict with industries or parties.

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