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The endoplasmic reticulum stress response is involved in apoptosis induced by aloe-emodin in HK-2 cells

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ABSTRACT

Aloe-emodin (AE; 1,8-dihydroxy-3-hydroxymethyl-9,10-anthracenedione) is one of the primary active compounds in total rhubarb anthraquinones (TRAs), which induce nephrotoxicity in rats. However, it is still not known whether AE has a similar effect on human kidney cells. In this study, 3-(4,5,-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays showed that AE decreases the viability of HK-2 cells (a human proximal tubular epithelial cell line) in a dose- and time-dependent manner. AE induced G2/M arrest of cell cycle in HK-2 cells, which was detected with propidium iodide (PI) staining. This apoptosis was further investigated by Hoechst staining, transmission electron microscopy (TEM), DNA fragmentation, and Annexin V/PI staining. Apoptosis of the cells was associated with caspase 3 activation, which was detected by Western blot analysis and a caspase activity assay. In addition, changes in the endoplasmic reticulum (ER) ultrastructure as observed by TEM showed the effects of AE on ER. Treatment with AE also resulted in an increase in eukaryotic initiation factor-2α (eIF-2α) phosphorylation, X-box binding protein 1 (XBP1) mRNA splicing, c-Jun N-terminal kinase (JNK) phosphorylation, glucose-regulated protein (GRP) 78 and CAAT/enhancer-binding protein-homologous protein (CHOP) accumulation. These results suggest that AE induces ER stress in HK-2 cells, which is involved in AE-induced apoptosis. In conclusion, AE induces apoptosis in HK-2 cells, and the ER stress is involved in AE-induced apoptosis *in vitro*. The implications of the toxic effects of AE for clinical use are unclear and these findings should be taken into account in the risk assessment for human exposure.

1. Introduction

Total rhubarb anthraquinones (TRAs), active therapeutic components that have been isolated from rhubarb roots (*Rheum palmatum* L. or *Rheum tanguticum* Maxim.), have been widely used in traditional Chinese medicine for centuries. Recent studies have demonstrated many pharmacological properties of rhubarb extracts, such as purgative (Peigen et al., 1984; Xu et al., 2011), anti-inflammatory (Cuellar et al., 2001; Gao et al., 2011), liver protection (Zhao et al., 2009), and attenuation of radiation-induced lung toxicity (Yu et al., 2008). Despite its therapeutic value, potential side effects of rhubarb extracts have been shown in experimental studies. It has been reported that TRAs induce nephrotoxicity that includes the swelling and denaturation of renal tubule epithelial cells in rats (Yan et al., 2006).

The toxicity of TRAs may result from anthraquinones and their derivatives, which are present in TRAs. Aloe-emodin (AE), one of these anthraquinone compounds, has been the subject of much recent investigation and was reported to exhibit a wide range of pharmacological activity, including anti-inflammatory (Das et al., 2011; Park et al., 2009), antimicrobial (Agarwal et al., 2000), and antiviral (Lin et al., 2008) properties. However, *in vivo* study has shown that AE may be the primary chemical component in rhubarb that causes hepatic and renal toxicity (Wang et al., 2009a).

Abbreviations: AE, Aloe-emodin; ASK1, apoptosis signal-regulating kinase 1; ATF, activating transcription factor; CHAC1, cation transport regulator-like protein 1; CHOP, CAAT/enhancer-binding protein-homologous protein; DR5, death receptor 5; eIF2α, eukaryotic initiation factor 2 alpha; ER, endoplasmic reticulum; FITC, fluorescein isothiocyanate; GADD153, growth arrest and DNA damage-inducible gene 153; GRP78, glucose-regulated protein 78; HK-2, human proximal tubular epithelial cell line; IRE1, inositol requiring 1; P-JNK, phosphorylated-c-Jun N-terminal kinase; MTT, 3-(4,5,-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI, propidium iodide; PERK, protein kinase-like ER kinase; PS, phosphatidylserine; TEM, transmission electron microscope; Tg, thapsigargin; TRA, total anthraquinone; TRAF2, tumor necrosis factor receptor-associated factor 2; TRB3, tribbles-related protein 3; TRITC, tetramethylrhodamineisothiocyanate; UPR, unfolded protein response; XBP1, X-box binding protein 1.

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Moreover, AE could induce primary damage to DNA in the cells of mouse livers and kidneys (Nesslany et al., 2009). These studies suggest that AE may have a cytotoxic effect on kidney cells.

Apoptosis plays a central role not only in the physiological processes of kidney growth and remodeling as well as in various human renal diseases and drug-induced nephrotoxicity (Servais et al., 2008; Wu et al., 2010). Apoptosis can be triggered by numerous stimuli. In addition, apoptosis is characterized by cell shrinkage, chromatin condensation, DNA fragmentation, phosphatidylserine (PS) externalization and the activation of specific caspases (Lam et al., 1999). Caspase 3 is one of the most important caspases for the execution of apoptosis in various cell types and is activated by initiator caspases, such as caspase 8 and 9, in response to pro-apoptotic signals (Lin et al., 2010; Patel et al., 1996). However, whether AE causes apoptosis of kidney cells and its possible mechanism remain unclear.

Endoplasmic reticulum (ER) stress has been proposed to be a mediator of apoptosis (Pallepati and Averill-Bates, 2011; Wang et al., 2011). The ER is responsible for the synthesis, folding, assembly, modification and transport of nascent proteins (Marciniak and Ron, 2006). The accumulation of misfolded proteins in the ER results in cellular stress, referred to as ER stress, and subsequently the unfolded protein response (UPR). In response to ER stress, three ER transmembrane proteins, including protein kinase-like ER kinase (PERK), inositol requiring 1 (IRE1) and activating transcription factor 6 (ATF6), are primarily activated (Lee et al., 2010; Verfaillie et al., 2010). Activation of the UPR induces an adaptative response to restore normal ER function. If the stress is prolonged or the adaptive response fails, apoptosis ensues (Malhotra and Kaufman, 2011; Oyadomari et al., 2002). However, the exact role of ER stress in AE-induced apoptosis is still undefined.

This study was designed to investigate if AE induces ER stress and apoptosis in HK-2 cells (a human proximal tubular epithelial cell line). Our results may help to clarify the risks to human health upon exposure to AE.

2. Materials and methods

2.1. Cell culture and chemicals

HK-2 cells (human proximal tubular cell line), kindly provided by Prof. Xueqing Yu (Department of Nephrology, The First Affiliated Hospital, Sun Yat-sen University, Guangdong, PR China), were cultured in Dulbecco modified Eagle medium/F12 (DMEM/F12, Gibco Laboratories, Grand Island, New York, USA) supplemented with 10% fetal bovine serum (FBS, Gibco), 50 units/mL of penicillin and 50 μg/mL of streptomycin. The cells were grown at 37 °C in a humidified incubator containing a 5% CO_2 atmosphere.

AE (CAS Registry No. 481-72-1, purity above 99%) was purchased from The National Institute for the Control of Pharmaceutical and Biological Products. It was dissolved in dimethylsulfoxide (DMSO) at a concentration of 100 mM and stored at −20 °C until use. DMSO (0.5%, v/v) was used as the control. An Annexin V/propidium iodide (PI) apoptosis detection kit was obtained from Keygen Biotech. Co. Ltd. (Nanjing, Jiangsu, China). Anti-caspase 3, anti-glucose-regulated protein (GRP)-78, anti-eukaryotic initiation factor-2α (eIF-2α), anti-phospho-eIF2α, and anti-CAAT/enhancer-binding protein-homologous protein (CHOP) antibodies were bought from Cell Signaling Technology Inc. (Beverly, MA, USA). The Hoechst 33258 staining and caspase 3 activity kit were obtained from Beyotime Institute of Biotechnology (Haimen, Jiangsu, China). Anti-phospho-c-Jun N-terminal kinase (JNK) antibody was purchased from New England Biolabs Inc. (Beverly, MA, USA). PI and anti-α-tubulin antibody were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other chemicals were analytical grade and obtained from commercial suppliers.

2.2. MTT assay

HK-2 cells were subcultured in a 96-well plate at an initial density of 2500 cells/well. After twenty-four hours, the cells were treated with fresh medium containing varying concentrations of AE (10, 25, 50, 75, and 100 μM) for 12, 24 and 48 h. DMSO (0.5%) was used as the control. Following treatment, 20 μL of a 5 mg/mL solution of the MTT tetrazolium substrate in phosphate buffered saline was added, and the solution was incubated for up to 4 h. The resulting violet formazan precipitate was solubilized by the addition of 200 μL of DMSO. After gently shaking at 37 °C for 10 min, the absorbance of the dissolved formazan grains within the cells was measured at 570 nm by a microplate reader (Thermo Multskan Ascent 354; Thermo Labsystems, Helsinki, Finland, USA).

2.3. Hoechst 33258 staining for apoptotic cells

HK-2 cells were grown on 15-mm glass coverslips in 6-well plates. The cells were treated with varying concentrations of AE (25, 50, and 100 μM) for 48 h or a single concentration of AE (100 μM) for various time periods (0, 12, 24, and 48 h). DMSO (0.5%) was used as the control. After treatment, the cells were washed three times with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 10 min. Subsequently, the fixed cells were washed three times with PBS and stained with Hoechst 33258 for 10 min at 37 °C. After washing three times with PBS, the glass coverslips were mounted, and fluorescence images were acquired with a fluorescence microscope (Olympus 1X71; Olympus, Tokyo, Japan). The condensation and fragmentation of nuclei was considered to be representative of apoptosis.

2.4. Transmission electron microscopy

Ultrastructural alterations in HK-2 cells caused by AE were examined using a transmission electron microscope (TEM). The cells were subcultured at 2×10^5 cells/well in a 6-well plate. After being treated with AE (100 μM) or the control (0.5% DMSO) for 48 h, the cells were collected and fixed with 2% glutaraldehyde in sodium cacodylate buffer for 1 h. The cells were washed and then post fixed with 1% osmium tetroxide for 30 min. Subsequently, the cells were washed with 0.2 M sodium cacodylate buffer (pH 7.4). The resulting brown-black pellet was embedded in 1% agarose at 4 °C overnight and then dehydrated with increasing serial concentrations (60%, 70%, 80% and 100%) of acetone for 30 min each. Low viscosity Spurr's epoxy group resin was then mixed with increasing serial concentrations of acetone. After infiltration with pure resin, the samples were transferred to silicone sample embedding molds and kept at 70 °C overnight. Before choosing areas for ultrastructural studies, semi-thick sections of 3 μm were cut on a Reichert Ultracut R microtome (Leica, Nussloch, Germany) and examined with a microscope. Ultrathin sections of 80 nm size were prepared and stained with 2% uranyl acetate and 1% lead citrate. Ultrathin sections were analyzed for ultrastructural alterations on a transmission electron microscope (Philips CM10, Netherlands).

2.5. DNA fragmentation assay

HK-2 cells were subcultured at 2×10^5 cells/well in a 6-well plate and then treated with the control (0.5% DMSO) or AE (25, 50, and 100 μM) for 48 h. After treatment, the cells were harvested and washed with PBS. Subsequently, the cells were lysed in 500 μL of lysis buffer (0.1 M NaCl, 10 mM Tris–HCl (pH 7.5), 10 mM EDTA, 0.5% SDS) for 1 h at 37 °C. The lysed cells were digested overnight with proteinase-K (500 μg/mL) at 55 °C, followed by incubation with 200 μg/mL RNase A at 37 °C for 2 h. DNA was extracted twice with phenol/chloroform (1:1; v/v) and precipitated at −20 °C with ethanol/sodium acetate. The DNA fragments were electrophoresed on 1.8% agarose gel. The gel was visualized under a UV lamp (UVItec Limited, Cambridge, UK).

2.6. Cell cycle analysis

The cell cycle distribution was analyzed using flow cytometry. The cells were subcultured at 2×10^5 cells/well in a 6-well plate. After treatment with the control (0.5% DMSO) or AE (25, 50, and 100 μM) for 48 h, the cells were harvested by trypsinization, washed twice with PBS, and fixed in 70% ethanol at −20 °C. The fixed cells were collected and resuspended in 500 μL of PBS with RNase A (100 μg/mL) at 37 °C for 1 h. Subsequently, the cell samples were incubated with PI (50 μg/mL) for 30 min. The DNA content of 2×10^4 cells for each sample was analyzed by Flow Cytometer (Becton Dickinson, San Jose, CA, USA). The data were analyzed with ModFit LT 2.0 software (Verity Software, Topsham, ME, USA).

2.7. Annexin V/PI staining assay

Apoptotic cells were analyzed with an Annexin V (fluorescein isothiocyanate (FITC)-conjugated)/PI apoptosis kit (Keygen Biotech, Nanjing, Jiangsu, China) using flow cytometry. The cells were subcultured at 2×10^5 cells/well in a 6-well plate. After treatment with varying concentrations of AE (25, 50, and 100 μM) for 48 h or the control (0.5% DMSO), the cells were harvested and resuspended in 500 μL binding buffer. Subsequently, the cells were incubated with 5 μL of Annexin V-FITC and 5 μL of PI (50 mg/mL) for 15 min in the dark. The cells were immediately analyzed by Flow Cytometer (Becton Dickinson, San Jose, CA, USA). For all the samples, 1×10^4 cells was acquired and analyzed with CellQuest software (Becton Dickinson, San Jose, CA). The percentage of cells residing in the lower right (early apoptotic cells) and upper right (late apoptotic/necrotic cells) region of the scatter plot of Annexin V-FITC was calculated for comparison.