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Effect of *Hydroxysafflor yellow A* on human umbilical vein endothelial cells under hypoxia

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ABSTRACT

Hydroxysafflor yellow A (HSYA), is a component of the flower, *Carthamus tinctorius* L. In this study, we investigated its effect on Human Umbilical Vein Endothelial Cells (HUVECs) under hypoxia. We evaluated cell viability using the MTT kit. The cell cycle distribution was analyzed by PI staining flow cytometric analysis. PI AnnexinV-FITC detection and the TUNEL assay were performed to evaluate the apoptosis rate. Nitric oxide (NO) generation in cell supernatant was measured by the Griess assay. RT-PCR, Western blot and Immunocytochemistry analysis were used to evaluate the changes of Bcl-2, Bax, p53 and eNOS. Our data showed that HSYA inhibited cell apoptosis and cell cycle G1 arrest induced by hypoxia. HSYA treatment increased the Bcl-2/Bax ratio of protein and mRNA, reduced p53 protein expression in cell nucleus. In addition, HSYA enhanced the NO content of cell supernatant under hypoxia, accompanied with upregulating eNOS mRNA expression and protein level. Taken together, these results demonstrate that HSYA could protect HUVECs from hypoxia induced injuries by inhibiting cell apoptosis and cell cycle arrest. These findings have partly revealed the molecular mechanism of HSYA on treating of ischemic heart disease. We expected our experiments might provide some clues for further research.

1. Introduction

The endothelium plays a predominant role in modulating many aspects of vascular homeostasis. Dysfunction of endothelial cell structure and function may contribute to the occurrence of diseases such as thrombosis and atherosclerosis. More and more evidence strongly suggests that vascular endothelial cells are impaired through apoptosis, contributing to the overall endothelial dysfunction in a range of clinic settings, including ischemic diseases (Scarabelli et al., 2002). Therefore, preventing endothelial cell apoptosis could result in improved endothelial function in patients with ischemia (Bruns et al., 2000). Consequently, understanding the pathways involved in vascular endothelial cell apoptosis and identifying strategies to inhibit this process would have important clinical implications.

In recent years, the clinical importance of herbal drugs has received considerable attention. The flower of the safflower plant, *Carthamus tinctorius* L., has been used extensively in Traditional Chinese Medicine for treatment of cardiovascular and cerebrovascular diseases. The extracts from *C. tinctorius* contain yellow and red pigments. *Hydroxysafflor yellow A* (HSYA) is the main chemical component of the safflower yellow pigments. Previous work reported that it could inhibit thrombosis and platelet aggregation (Tian et al., 2003), reduce the myocardial infarct size (Wang et al., 2006). and exert beneficial actions in congestive cardiac failure rats by suppressing ET-1, iNOS and oxidative stress in infarcted tissue(He et al., 2008). Liu et al. (2008) reported that HSYA protected the myocardium against ischemia–reperfusion injury by inhibiting mitochondrial permeability transition pore opening. However, the exact mechanism of the cardiovascular-protective effect of HSYA is still poorly understood. Accordingly, we explore the role of HSYA in apoptosis induced by hypoxia in vascular endothelial cells.

2. Materials and methods

2.1. Materials

The yellow pigment extract from *C. tinctorius* containing hydroxysafflor yellow A (HSYA) was generously provided by Dr. Gui Sheng Li, Shandong Natural Drugs Research & Development Center, province Shandong, China. HSYA was further purified by Professor Pei Cheng Zhang, Department of natural products chemistry, Institute of Materia Medica, Chinese Academy of Medical Sciences, Beijing, China (Fig. 1). This drug was identified by spectroscopy and its purity (>98%) was determined by HPLC. Its molecular formula is C_{27} $H_{32}O_{16}$ with a molecular weight of 611.16 Daltons. It is soluble in water, with a pH

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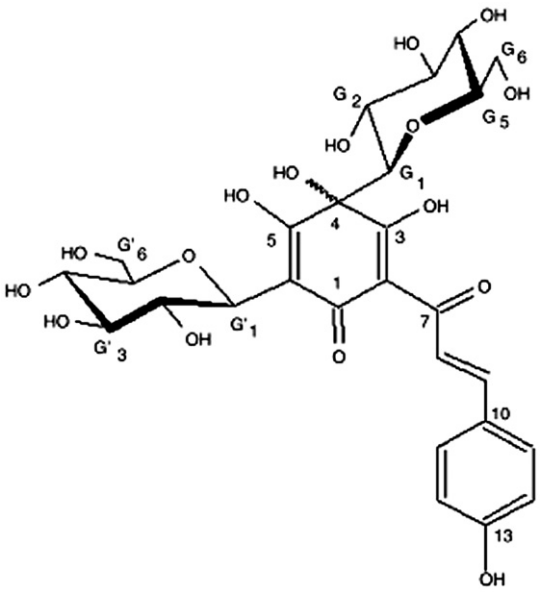

Fig. 1. The chemical structure of hydroxysafflor yellow A.

about 5. Medium 199 was obtained from Invitrogen Corporation, USA; FBS (Fetal Bovine Serum) from HyClone Corporation, USA; endothelial cell growth factor, MTT, RNase from Sigma, USA; Annexin V-FITC/PI kit from Peking University Center for Human Disease Genomics, Beijing, China; and In Situ Cell Death Detection Kit, POD kit from Roche, Applied Science, Germany; Antibodies to Bcl-2, Bax, P53, eNOS were purchased from Santa Cruz Biotechnology, USA; Immunohistochemistry kit from Zhongshan Goldenbridge Biotechnology, China; TRIzol and ThermoScript RT-PCR kit were purchased from Invitrogen Corporation, USA. NO assay kit was purchased from Nanjing Jiancheng Bioengineering Institute, China.

2.2. Culture of human umbilical vein endothelial cells (HUVECs) and induction of hypoxia

HUVECs were isolated from fresh human umbilical veins as described with some modifications (Galley and Webster, 2004). Briefly, human umbilical veins were flushed with phosphate-buffered saline (PBS), then filled with PBS containing 0.2% collagenase type II and incubated for 10 min at 37 °C. The HUVECs were removed from the vein by PBS wash. The primary isolated HUVECs were maintained in 2% gelatin-coated tissue culture plates in complete growth medium 199 supplemented with 20% fetal bovine serum, 10 μg/ml endothelial cell growth factor, 10 mg/ml heparin, and penicillin-streptomycin (50 mg/ml each) at 37 °C in a 5% CO2 incubator. HUVECs from passages 3–4 were used in this study. The endothelial phenotype was confirmed using phase-contrast microscopy and staining for the endothelial-specific von Willebrand factor. Umbilical cords were obtained by written consent, with approval from the local ethics committee.

For cell culture experiments, cells were either exposed to normoxia (21% O_2, 5% CO_2) or placed in a hypoxic incubator with 1% O_2, 94% N_2, 5% CO_2 (Forma Series II Water Jacketed CO_2 incubator, Thermo electron corporation).

2.3. Cell viability

MTT assay is a standard method used to assess cell viability, which measures mitochondrial activity in viable cells (Hansen et al., 1989). HUVECs were seeded in 96 multi-well plates at 3×10^4 cells/well in 200 μl of medium containing 10% FBS. The cells were allowed to attach for 24 h, the medium was changed with fresh medium supplemented with HSYA at concentrations of 1×10^{-6} M, 1×10^{-5} M, 1×10^{-4} M and cells were transferred to a standard incubator or to a hypoxia chamber for 12 h, 24 h, 48 h respectively. MTT was added to the cultures at a terminal concentration of 1 mg/ml and incubated for another 4 h at 37 °C. The supernatant was carefully aspirated, and 200 μl DMSO was added to dissolve formazan. The 96 well microplate was then transferred to the microplate reader for measuring the absorption at 570 nm. The absorbance was used as a measurement of cell viability, normalized to cells incubated in control medium which were considered 100% viable.

2.4. Annexin-V-FITC labeling and fluorescence-activated cell sorting analysis (FACS)

For FACS analysis, cultured cells were exposed to FITC-conjugated annexin V and to the fluorescent dye propidium iodide (PI). The cells were harvested, washed with ice-cold PBS, and resuspended with annexin V-fluorescein isothiocyate (FITC) and PI according to the protocol of the annexin V-FITC kit. The final mixture of annexin V-FITC/PI stain was then applied to a FACScalibur flow cytometer and CellQuest software for analysis (approximately 10,000 events). The results were interpreted as follows: cells negative for both PI and annexin-V-FITC staining were considered live cells; PI-negative, annexin-V-FITC-positive stained cells were considered in early apoptosis; PI positive, annexin-V-FITC-positive-stained cells were considered in late apoptosis or secondary necrosis.

2.5. Terminal deoxynucleotidyl transferase-meditated dUTP nick end-labeling (TUNEL) assay for DNA apoptotic fragmentation

The terminal deoxynucleotidyltransferase-mediated dUTP end labelling (TUNEL) assay was used to identify cell apoptosis. HUVECs were seeded onto coverslips at a density of 2×10^4 cells, grown overnight, then subjected to hypoxic or normoxic conditions in the presence or absence of HSYA (1×10^{-6}, 1×10^{-5}, 1×10^{-4} M) for 48 h. Fragmented DNA staining of apoptotic cells was carried out using a commercial kit. Briefly, the cells were rinsed twice with pre-warmed PBS followed by fixation using 4% paraformaldehyde at 4 °C. The fixed cells were permeabilized with 20 μg/ml protease K in TBS and endogenous peroxidase was inactivated by 3% H_2O_2 in methanol. The cells were rinsed again with PBS and incubated with 50 μl per sample of TUNEL reaction mixture for 1 h at 37 °C. A negative control was included in each staining where only the labelling solution was added. The slides were then incubated with streptavidin horseradish peroxidase conjugate, followed by incubation with 3, 3 - diaminobenzidine. Apoptotic cells were identified by their dark brown nuclei seen under a light microscope.

2.6. Cell cycle analysis

The HUVECs were seeded into 60 mm culture dishes at 5×10^5 cells/ml and cultured until attached. The cells were serum starved for 12 h with the serum-free medium M199, followed by fresh media change containing 10% FBS with or without HSYA (1×10^{-6}, 1×10^{-5}, 1×10^{-4} M) and incubated under normoxia or hypoxia for 48 h. Cell cycle distribution was evaluated by propidium iodide staining of nuclei and flow cytometric analysis (Li et al., 1997). Alternatively, pelleted cells were washed twice with PBS and then fixed in 70% cold ethanol overnight at −20 °C. After washing again the cells were re-suspended in PBS containing RNase A (200 μg/ml), and incubated at 37 °C for 30 min. Propidium iodide was added to the cell suspensions to a final concentration of 100 μg/ml. The fluorescence intensity of PI was analyzed with a FACScalibur flow cytometer and CellQuest software.

2.7. Nitric oxide (NO) release assay

HUVECs were cultured after the procedure as described in the cell viability. After incubation, the supernatant was collected from plates