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Tanshinone IIA reduces apoptosis induced by hydrogen peroxide in the human endothelium-derived EA.hy926 cells

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ABSTRACT

Ethnopharmacological relevance: Salvia Miltiorrhiza Bunge (also known as herb Danshen in Chinese) is a widely used Chinese herbal medicine. Tanshinone IIA (TSN IIA) is considered to be the most important bioactive ingredient in Danshen and exhibits an anti-atherosclerotic activity.

Aim of study: To evaluate the protective effect of TSN IIA on the human endothelial EA.hy926 cells injured by hydrogen peroxide in vitro and its possible mechanism.

Materials and methods: The EA.hy926 cells were incubated for 24 h with different concentrations of TSN IIA (5, 10 and 20 μg/μL) or DMEM. Subsequently, cells were treated with 300 μmol/L H_2O_2 for another 4 h. Then, the percentage of cell viability was evaluated by 3-(4, 5-di-methylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. The apoptosis of EA.hy926 cells was detected by flow cytometry with AnnexinV-FITC/PI double staining and laser scanning spectral confocal technique. The generation of intracellular reactive oxygen species (ROS) generation was analyzed by flow cytometry. The mRNA expressions of caspase-3, Bcl-2 and Bax were tested by real time-reverse transcription polymerase chain reaction (real time RT-PCR). The protein expression of Bcl-2 and Bax was determined by Western blotting. MDA levels, NO production, LDH leakage, and SOD as well as caspase-3 activities were also measured using standard methods.

Results: Loss of cell viability and excessive cell apoptosis were observed in EA.hy926 cells after 4 h of challenge with H_2O_2 (300 μmol/L). However, cell apoptosis was attenuated in different concentrations of TSN IIA (5, 10 and 20 μg/μL) pretreated cells. Furthermore, TSN IIA markedly inhibited the elevation of ROS evoked by H_2O_2. Real time RT-PCR and Western blotting analysis showed that TSN IIA significantly decreased the expressions of pro-apoptotic proteins (Bax and caspase-3) while significantly increased the expression of anti-apoptotic protein Bcl-2, and resulted in obvious reduction of Bax/Bcl-2 ratio in EA.hy926 cells induced by H_2O_2.

Conclusion: These observations provide preliminary evidence that TSN IIA protects EA.hy926 cells against H_2O_2 damage, which is mainly associated with the ROS generation, followed by the imbalance of the Bax/Bcl-2 ratio, and caspase-3 activation leading to apoptosis.

1. Introduction

Endothelial injury is considered to be an initial step in the pathogenesis of atherosclerosis (Higashi et al., 2009), which is also the pathological basis of various cardiovascular and cerebrovascular disorders (Katz et al., 2001). Growing evidence reveals a relationship between oxidative stress and endothelial function and oxidative stress has been recognized as a key mechanism in the development of vascular damage, particularly atherosclerosis (Minuz et al., 2006). There are several possible mechanisms for the oxidative stress impairment of endothelial function in cardiovascular diseases, including enhanced production of reactive oxygen species (ROS) and decreased release of

Abbreviations: TSN IIA, Tashinone IIA; MTT, 3-(4, 5-di-methylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide; ROS, reactive oxygen species; Real time RT-PCR, real time-reverse transcription polymerase chain reaction; NO, nitric oxide; H_2O_2, hydrogen peroxide; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; SOD, super-oxide dismutase; LDH, lactate dehydrogenase; MDA, malonaldialdehyde; PI, propidium iodide

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nitric oxide (NO), as well as an attenuated antioxidant system (Cai and Harrison, 2000; Deanfield et al., 2007). Oxidative stress may result in apoptosis of endothelial cell, which contributes to atherogenesis and other vascular diseases (Harrison et al., 2003; Quagliaro et al., 2003; Sudoh et al., 2001). A number of investigators have reported that apoptosis of endothelial cell can be triggered by ROS (Irani, 2000; Li and Shah, 2004). Furthermore, apoptotic cell death following injury of vascular endothelium is assumed to play an important role in the pathogenesis of atherosclerosis (Cines et al., 1998; Falk, 2006).

Tanshinone IIA (TSN IIA) is the most abundant diterpene quinone in *Salvia Miltiorrhiza Bunge* (Danshen). It is a widely prescribed traditional herbal medicine used for the prevention and treatment of atherosclerosic disease (Wang et al., 2003, 2010). It is also considered to be the most important bioactive ingredient in Danshen and exhibits a variety of cardiovascular and cerebrovascular activities. TSN IIA provides beneficial effects toward atherosclerosic disease through several pathways. Accumulating studies demonstrated that TSN IIA possesses many biological and pharmacologic properties primarily depending on its anti-oxidative effects (Lin et al., 2006; Tang et al., 2007). Although TSN IIA has been proved to have anti-oxidant effects on preventing endothelial cell from oxidative stress-triggered damage and apoptosis (Lin et al., 2006; Wu et al., 2007), many of its anti-oxidant and anti-apoptotic mechanisms remain to be demonstrated. And little data is available about its anti-atherosclerotic role and mechanisms in human endothelium-derived EA.hy926 cells. In the present study, we examined the protective effects of TSN IIA on hydrogen peroxide (H_2O_2)-induced apoptosis of EA.hy926 cells and investigated the possible mechanisms of action involved.

2. Materials and methods

2.1. Drug and reagents

TSN IIA was purchased from the Chinese Institute for Drug and Biological Product Control (Beijing, China) and then was dissolved in DMSO (final concentration 0.2 mL/L). The solution was filtered through a 0.22 μm micropore filter and stored at 4 °C. DMEM medium, trypsin, and fetal bovine serum (FBS) were purchased from Hyclone (Hyclone Logan, UT). 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), dimethyl sulphoxide (DMSO), penicillin, streptomycin were obtained from Sigma (St. Louis, MO, USA). Rabbit anti-human Bcl-2 antibody, rabbit anti-human Bax antibody and goat anti-rabbit IgG were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Super-oxide dismutase (SOD), NO, lactate dehydrogenase (LDH), malonaldialdehyde (MDA) assay kits, H_2O_2, Annexin V fluorescein isothiocyanate (FITC) and propidium iodide (PI) apoptosis detection kits were produced by Nanjing Key-Gen Biotech Co., Ltd. (Nanjing, China). The SYBR Exscript™ RT-PCR Kit and Trizol reagent were purchased from TaKaRa Bio Inc. (Dalian, China). RIPA Lysis Buffer, caspase-3 activity kit, ROS assay kit was produced by Beyotime Institute of Biotechnology (Nantong, China). All other reagents commercially available were of the highest purity.

2.2. Cell culture

EA.hy926 human vascular endothelial cell line was purchased from American Type Culture Collection and maintained in high-glucose DMEM medium containing 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C, 5% CO_2. Cells in logarithmic growth phase were used for further experiments. Before experimental intervention, confluent-cultured cells were preincubated for 24 h in serum-starved medium including DMEM with 1% fetal bovine serum.

2.3. Oxidative damage induced by H_2O_2

EA.hy926 cells were cultured in 24-well plates at a density of 2×10^5 cells/ml for 24 h. TSN IIA (5, 10 and 20 μg/μL) was dissolved in DMSO (no more than 0.1% in v/v) and was added into the wells for 24 h incubation, and then the cells were exposed to 300 μmol/L H_2O_2 for another 4 h except the normal control.

2.4. Measurement of cell viability

The viability of EA.hy926 cells was measured by a colorimetric assay using MTT. The assay was performed by seeding EA.hy926 cells in the concentration of 1×10^4 cells/well in 96-well plate. After different concentrations of TSN IIA (5, 10 and 20 μg/μL) in DMEM medium were added into the wells to incubate 24 h, cells were exposed to 300 μmol/L H_2O_2 for further 4 h. Then MTT solution (5 mg/mL) was added to each well, and the plate was incubated 4 h at 37 °C. After incubation, medium was removed and 150 μL DMSO was added to each well for formazan solubilization. Finally, the optical density (OD) of each well was measured on a microplate reader at 570 nm. The OD of formazan formed in untreated cells was taken as 100% viability.

2.5. Measurement of intracellular ROS

Intracellular ROS formation was quantified using redox-sensitive dye 2,7-dichlorodihydrofluorescin diacetate (DCFH-DA) and flow cytometric analysis. Briefly, after exposure to 300 μmol/L H_2O_2 for 4 h, cells were harvested with trypsin and washed with phosphate buffered saline (PBS) twice. Then, 1×10^6 cells were incubated with 10 μmol/L DCFH-DA for 20 min at 37 °C. The fluorescence of 2,7-dichlorofluorescein (DCF) was detected using flow cytometry and all the measurements were repeated in triplicate.

2.6. Determination of NO production and LDH leakage

The production of NO was tested by measuring the accumulation of nitrites in the supernatant of cells. In brief, 100 μl Griess reagent was added to 100 μl of sample. After incubating at room temperature for 10 min, the OD value was measured at 550 nm with a Microplate Reader. To evaluate the effects of TSN IIA on LDH leakage, EA.hy926 cells were treated in preparation for a cell viability assay. At the end of incubation, the supernatant was collected and LDH leakage was measured using the assay kit according to the manufacturer's instructions.

2.7. Evaluation of MDA levels and SOD activities

After exposured to 300 μmol/L H_2O_2 for 4 h, EA.hy926 cells were collected to measure MDA levels and SOD activities. Briefly, cells were washed twice with PBS and lysed with lysis buffer. Then the homogenate was centrifuged at 12,000*g* at 4 °C for 15 min. The MDA level and SOD activities in the supernatant were measured by spectrophotometric methods.

2.8. Determination of apoptotic cells

Double staining for Annexin V-FITC and propidium iodide (PI) was performed to estimate the apoptotic rate of EA.hy926 cells. Briefly, after incubated with various concentrations of TSN IIA for 24 h, EA.hy926 cells were treated with 300 μmol/L H_2O_2 for another 4 h.