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A novel compound derived from danshensu inhibits apoptosis via upregulation of heme oxygenase-1 expression in SH-SY5Y cells

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ABSTRACT

Background: Heme oxygenase-1 (HO-1) has potential anti-apoptotic properties. A novel compound [4-(2-acetoxy-3-((R)-3-(benzylthio)-1-methoxy-1-oxopropan-2- ylamino)-3-oxopropyl)-1,2-phenylene diacetate (DSC)] was synthesized by joining danshensu and cysteine through an appropriate linker. This study investigated if the cytoprotective properties of DSC involved the induction of HO-1.
Methods: We evaluated the cytoprotective effects of DSC on H_2O_2-induced cell damage, apoptosis, intracellular and mitochondrial reactive oxygen species (ROS) production, mitochondrial membrane potential ($\Delta\Psi_m$) loss, and apoptosis-related proteins expression and its underlying mechanisms.
Results: DSC concentration-dependently attenuated cell death, lactate dehydrogenase release, intracellular and mitochondrial ROS production, and $\Delta\Psi_m$ collapse, modulated apoptosis-related proteins (Bcl-2, Bax, caspase-3, p53, and cleaved PARP) expression, and inhibited phosphorylation of extracellular signal-regulated kinase 1/2 in SH-SY5Y cells induced by H_2O_2. In addition, DSC concentration-dependently induced HO-1 expression associated with nuclear translocation of nuclear factor-erythroid 2 related factor 2 (Nrf-2), while the effect of DSC was inhibited by a phosphoinositide 3-kinase (PI3K) inhibitor LY294002. Furthermore, the protective effect of DSC on H_2O_2-induced cell death was abolished by HO-1 inhibitor ZnPP, but was mimicked by carbon monoxide-releasing moiety CORM-3 or HO-1 by-product bilirubin. Finally, DSC inhibited H_2O_2-induced changes of Bcl-2, Bax, and caspase-3 expression, and all of these effects were reversed by HO-1 silencing.
Conclusions: Induction of HO-1 may be, at least in part, responsible for the anti-apoptotic property of DSC, an effect that involved the activation of PI3K/Akt/Nrf-2 axis.
General significance: DSC might have the potential for beneficial therapeutic interventions for neurodegenerative diseases.

1. Introduction

The neurodegenerative disorder is characterized by progressive degeneration and loss of neurons in the brain, although pathological mechanism remains uncertain [1]. Oxidative stress occurs when reactive oxygen species (ROS) were generated in excess through the reduction of oxygen [2]. Oxidative stress was implicated in the pathogenesis of neurological disorders, such as dysautonomia, Alzheimer's disease, Parkinson's disease, and stroke [2,3]. ROS, such as superoxide and hydrogen peroxide (H_2O_2), were considered as major causes of neuronal cell death by dysregulation of signaling pathways and/or by oxidative damage to cellular macromolecules [3,4]. Therefore, pharmacological approaches for intervening in oxidative stress might be therapeutic intervention strategies for neurodegenerative disorders [4,5].

Two genetically distinct isoforms of heme oxygenase (HO) have been described: an inducible form HO-1 and a constitutively expressed form HO-2 [6]. Whereas HO-2 regulated normal physiological cell function; HO-1, also known as heat-shock protein 32 or inducible HO, was induced by a wide variety of stimuli or conditions and upregulation of HO-1 in neurons was strongly protective against oxidative damage and cell death [7,8]. HO-1 catalyzed the degradation of heme to biliverdin, carbon monoxide (CO) and ferrous iron, with biliverdin being subsequently converted to bilirubin [6]. Biliverdin and bilirubin were the most potent endogenous ROS scavengers [6]. CO was also an efficient cytoprotective mediator in oxidant-induced apoptosis in neurons [9]. The transcription factor nuclear factor-erythroid 2 related factor 2 (Nrf-2) played a crucial role in antioxidant response element-mediated expression of HO-1. Under basal conditions, the Kelch-like ECH-associated protein 1 (Keap1)

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banded to Nrf-2 in the cytoplasm, in the presence of stimulus or inducing agents lead to disruption of the Keap1–Nrf2 complex thereby allowing Nrf-2 translocation into nucleus, triggering the simultaneous expression of numerous protective enzymes, including HO-1 [10]. Thus, pharmacological activation of Nrf-2 and subsequent modulation of HO-1 expression were considered important molecular targets for therapeutic intervention [11,12].

Salvia miltiorrhiza Bunge, a traditional Chinese herbal medicine, has been widely used as a therapeutic agent for neurodegenerative disorders for hundreds of years [10,13]. *S. miltiorrhiza* bunge contains two types of bioactive constituents, hydrophilic phenolic (danshensu, salvianolic acid B, etc) and lipophilic quinines (tanshinone I, tanshinone IIA, dihydrotanshinone I, etc) [14]. Danshensu (3-(3',4'-dihydroxyphenyl)-(2R)-lactic acid) might reduce the risk for neurodegenerative disorder associated with its anti-inflammatory and anti-apoptotic effects [14,15]. Due to the chemical instability of phenolic hydroxyl groups of danshensu and its low content in *S. miltiorrhiza* Bunge, the chemical stability of danshensu derivatives were synthesized asymmetrically and exerted cardioprotective effects by modulating the expression of apoptosis-related proteins and by inhibiting lipid hyperoxidation [16]. In addition, our recent studies demonstrated that L-cysteine derivative S-propargyl-cysteine exerted various salubrious biological activities, including anti-inflammatory [17,18], anti-oxidative [19], and anti-apoptotic activities [20]. In order to obtain a polyvalent drug, we chemically synthesized a series of novel amide and thioester conjugates of danshensu–cysteine derivatives by joining two drugs through an appropriate linker or bond in the light of the guidance of medicinal chemical hybridization [21]. Among them, a novel conjugate DSC (Fig. 1A) was demonstrated promising neuroprotective bioactivities and was selected to study further to probe into its mechanism.

This study was then conducted to evaluate the role and possible mechanisms of DSC on apoptosis in H_2O_2-stimulated human neuroblastoma SH-SY5Y cells. Our results demonstrated that DSC attenuated H_2O_2-induced apoptosis via the preservation of mitochondrial function in SH-SY5Y cells. To our knowledge, this was also the first study that induction of HO-1 might be, at least in part, responsible for the anti-apoptotic property of DSC, an effect that involved nuclear translocation of Nrf2 and activation of the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway.

2. Materials and methods

2.1. Reagents and antibodies

2 ,7 -dichlorodihydrofluorescein diacetate (H_2DCF-DA), dihydroethidium (DHE), 5,5′, 6,6′-tetrachloro-1,1′, 3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), and MitoSOX™ Red were from molecular probes (Eugene, OR, USA). Dulbecco's modified Eagle's medium (DMEM)/F-12 medium and fetal bovine serum (FBS) were from GIBCO-BRL (USA). LY294002, PD98059, and SB203580 were from Calbiochem (San Diego, CA). Antibodies against total- and phosphor (p)- c-Jun N-terminal kinase 1/2 (JNK1/2) (Thr^{183} and Tyr^{185}), total- and p-p38 (Thr^{180} and Tyr^{182}), total- and p-ERK1/2 (Thr^{202}/Tyr^{204}), total- and p-Akt (Ser^{473}), p53, and caspase-3 were purchased from Cell Signaling Technology (Danvers, MA). Antibodies against β-actin, Bax, Bcl-2, HO-1, and Nrf-2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against Lamin A/C and cleaved-poly (ADP-ribose) polymerase (PARP) were from Epitomics (Burlingame, CA). $[Ru(CO)_3Cl_2]_2$ (CORM-3), bilirubin, zinc protoporphyrin IX (ZnPP), H_2O_2, and all other chemicals used in this study were purchased from Sigma (St. Louis, MO). Danshensu and DSC were provided by Prof. Yang Wang (Department of Medicinal Chemistry, School of Pharmacy, Fudan University) and purity was over 99% determined by high performance liquid chromatography. Danshensu and DSC were dissolved in distilled water and dimethyl sulfoxide (DMSO), respectively. The final concentration of DMSO was less than 0.1%.

2.2. Cell culture and treatment

Human neuroblastoma SH-SY5Y cells were from ATCC (Manassas, VA) and maintained in DMEM/F-12 containing 10% FBS and maintained at 37 °C with 95% humidified air and 5% CO_2. In some experiments, the cells were preincubated with DSC (5-100 μM, 4 h), N-acetyl-L-cysteine (NAC, 5 mM, 4 h), CORM-3 (100 μM, 1 h), bilirubin (20 μM, 1 h), ZnPP (HO-1 inhibitor, 10 μM, 1 h), SB203580 (p38 inhibitor, 10 μM, 1 h), LY294002 (PI3K inhibitor, 10 μM, 1 h), SP60012 (JNK1/2 inhibitor, 10 μM, 1 h), or PD98059 (ERK1/2 inhibitor, 10 μM, 1 h). Subsequently, stimulated with H_2O_2 (200 μM) for indicated periods to measure phosphorylation of mitogen-activated protein kinases (MAPK) (p38, ERK1/2, and JNK1/2), mitochondrial membrane potential ($\Delta\Psi_m$), intracellular and mitochondrial ROS production, apoptosis-related proteins and HO-1 expression, cell viability, lactate dehydrogenase (LDH) release, and apoptotic ratio.

2.3. Cell viability assay

Cell viability was evaluated by 3-(4, 5-dimetrylthiazol)-2, 5-diphenyltetrazolium bromide (MTT) assay as described previously [22]. In brief, the medium was changed before the assay. MTT dissolved in phosphate buffered saline (PBS), was added to the culture medium to reach a final concentration of 0.5 mg/ml. After incubation at 37 °C for 4 h, the culture media containing MTT were removed. DMSO was then added into each well, and the absorbance at 570 nm was measured by a microplate reader (TECAN M200, Austria GmbH, Austria).

2.4. LDH release

SH-SY5Y cells in 6-well plates were pretreated with DSC or NAC for 4 h, then stimulated with H_2O_2 (200 μM) for 12 h, LDH release in the supernatants was detected by a commercially available kit (Jiancheng Biotechnology, Nanjing, China) according to the manufacturer's instructions. The LDH release was quantified by measuring the wavelength absorbance at 490 nm with a microplate reader (TECAN M200, Austria GmbH, Austria).

2.5. Nuclear staining with Hoechst 33258

SH-SY5Y cells (1×10^3 cells/well) in 24-well plates were pretreated with or without DSC or NAC for 4 h, and then stimulated with H_2O_2 for the 12 h. The nuclear morphological change of apoptotic cells was investigated using the Hoechst 33258 nuclear staining kit (Beyotime Biotechnology, Nanjing, China) according to the manufacturer's instructions. The nuclear morphology was observed under a fluorescence microscope (Carl Zeiss) using excitation/emission of 340/460 nm.

2.6. Intracellular and mitochondrial ROS measurement

SH-SY5Y cells in 24-well plates were pretreated with DSC and NAC for 4 h. After removing the DSC and NAC from the wells, the cells were incubated with H_2DCF-DA (10 μM) or DHE (10 μM) for 30 min. Then stimulated with H_2O_2 (200 μM) for 2 h, and the fluorescence intensity of H_2DCF-DA and DHE was measured/detected by a fluorescence spectrophotometer (M1000, TECAN, Austria GmbH, Austria) using excitation/emission of 485/530 nm and a fluorescence microscope (Carl Zeiss) using excitation/emission of 535/610 nm, respectively. All values for each treatment group have been normalized to the control group.

MitoSOX Red was used to measure mitochondrial ROS production. SH-SY5Y cells were pretreated with DSC and NAC for 4 h. After removing the DSC and NAC, the cells were incubated with MitoSOX (5 μM) in Hanks Balanced Salt Solution for 30 min. Then stimulated with H_2O_2 (200 μM) for 2 h, followed by 3 washes with Hanks Balanced Salt Solution. Samples