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Original article

Activation of Akt and JNK/Nrf2/NQO1 pathway contributes to the protective effect of coptisine against AAPH-induced oxidative stress

Yin-Ran Hu[a,c,d], Hang Ma[a,c,d], Zong-Yao Zou[a,c,d], Kai He[a,c,d], Yu-Bo Xiao[a,c,d], Yue Wang[b], Min Feng[a,c,d], Xiao-Li Ye[b], Xue-Gang Li[a,c,d,*]

[a] College of Pharmaceutical Sciences, Southwest University, Chongqing, 400716, China
[b] School of Life Sciences, Southwest University, Chongqing, 400715, China
[c] Chongqing Productivity Promotion Centre for the Modernization of Chinese Medicine, Chongqing, 400716, China
[d] Chongqing Engineering Research Center for Pharmaceutical Process and Quality Control, Chongqing, 400716, China

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ABSTRACT

Coptisine (COP) is one of the main active constituents of *Coptidis Rhizoma*. Previous studies have clarified that COP possesses antioxidant activity, but its defensive effects against pathological characteristics accompanied by oxidative damage in animal models and antioxidant mechanism are still unclear. Therefore, our purpose was to confirm the antioxidant activity of COP and explore its mechanism of action. We first detected the effects of COP on intracellular reactive oxygen species (ROS), heart beating rate, lipid peroxidation and cell death in zebrafish model with AAPH-induced oxidative stress. The results showed that COP of 10 μg/mL significantly reduced ROS production, the increase of heart beating rate, lipid peroxidation and cell death by 41.3%, 24.5%, 26.5% and 30.0%, respectively. In addition, COP of 0.8 μg/mL also decreased ROS, increased glutathione (GSH) content and elevated activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) by 40.1%, 19.8%, 18.3% and 49.3%, respectively in HepG2 cells. Further assays were carried out to explore the mRNA expression in zebrafish and protein expression of key factors in HepG2 cells. We demonstrated that COP up-regulated phase II antioxidant enzymes NAD(P)H/quinone oxidoreductase 1 (NQO1) through activating the nuclear factor erythroid-2 related factor 2 (Nrf2). Moreover, as the upstream signalings of Nrf2, the protein kinase B (Akt) and c-Jun NH2-terminal kinase (JNK) signalings were also induced by COP. And up-regulating Nrf2-mediated NQO1 expression of COP was in Akt and JNK-dependent manner. Taken together, COP exerted its antioxidant activity against AAPH-induced toxicity involving in activating Akt and JNK/Nrf2/NQO1 pathway.

1. Introduction

Reactive oxygen species (ROS) includes superoxide anion ($O_2^{-\bullet}$), hydroxyl radical ($OH^{\bullet}$), as well as some non-free radicals like hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) [1]. The producing and scavenging of ROS are dynamically balanceable and have important signaling functions within the cell under normal condition [2,3]. However, when harmful stimulations such as radiation [4], environmental pollution [5], petrochemical products [6], heavy metal [7] and chemical inducers act on the body, excess ROS production from mitochondria would exceed the scavenging ability of antioxidant defense system, and then the result is oxidative stress [8]. The oxidative stress can leads biomolecules, including DNA, membrane lipids, enzymes, and structural proteins to be damaged because of being oxidized to an extent in which they cannot be repaired [9]. Oxidative stress also accounts for a series of human diseases threatening global health, such as cardiac vascular disease [10,11], nervous system disease [12,13], cancers [14], and aging [15]. Therefore, antioxidant defense is crucial to the initiation and development of these chronic diseases.

Coptisine (COP), derived from traditional Chinese medicine *Coptidis Rhizoma*, is a kind of original berberine type alkaloids with isoquinoline ring structure (Fig. 1). It presents multiple pharmacological activities, including antifungal, gastric mucosa protection [16], anti-hypercholesterolemia [17], anti-hypertriglyceridemia, anti-obesity [18] and antioxidant [19]. Among them, antioxidant activity had been mainly reported *in vitro* studies of scavenging free radicals and to make a primary evaluation of changes of

* Corresponding author at: College of Pharmaceutical Sciences, Southwest University, # 2, Tiansheng Road, Beibei, Chongqing 400716, China.
E-mail address: xuegangli@swu.edu.cn (X.-G. Li).

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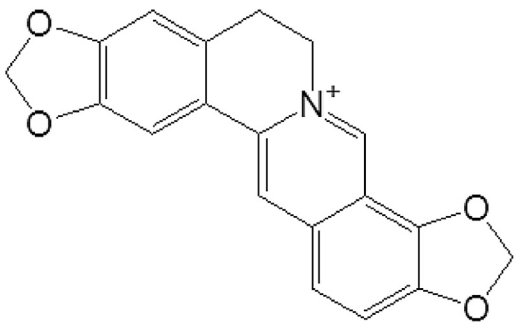

Fig. 1. The chemical structure of coptisine.

oxidative stress markers as well as antioxidant enzymes *in vivo*. So, we aimed to further investigate its antioxidant activity and explore the underlying mechanism.

Numerous studies have showed that the nuclear factor Nrf2 pathway is involved in the mechanism of antioxidant. Nrf2 could increase the transcription of its target genes, which included antioxidant enzymes NQO1 and it was also regulated by upstream kinases, like Akt or the mitogen-activated protein kinases (MAPKs) [20,23]. More importantly, as an isoquinoline alkaloid, berberine also derived from *Coptidis Rhizoma* is with similar structure to that of COP. Previous reports indicated berberine activated Akt and MAPKs signalings, and then induced Nrf2-mediated expression of antioxidant enzymes, like NQO1 [24]. So, we regarded Akt and MAPKs/Nrf2/NQO1 as our research pathway in mechanism research of COP antioxidant. Thus, our work was conducted to investigate the potential protective effect of COP on AAPH-induced oxidative stress and further explore its mechanism of action.

2. Materials and methods

2.1. Materials and reagents

Standard substance of COP was purchased from Chengdu Must Biotechnology Co., Ltd. (Chengdu, China). COP was synthesized according to previous methods in our lab with its structure confirmed by nuclear magnetic resonance spectrum diagram and the purity of COP is 98.5% [25]. L-ascorbic acid (Vitamin C, Vit. C) and 3-(4,5-Dimethylthiazol-z-yl)-3,5-diphenyltet- razolium bromide (MTT) were purchased from Beijing Dingguo Changsheng Biotechnology Co., Ltd. (Beijing, China). 2, 2′-azobis [2-methyl-propionamidine] dihydrochloride (AAPH) was purchased from Xiya Chemical Industry Co., Ltd. (Shandong, China). 2′, 7′-Dichlorofluorescin diacetate (DCFH-DA) was from Sigma-Aldrich, Co. (St. Louis, USA). 1, 3-Bis (diphenylphosphino)-propane (DPPP) was from TCI (shanghai) development Co., Ltd. (Shanghai, China). Acridine orange (AO) was from Gen-View Scientific Inc. (El Monte, CA, USA). Commercial antioxidation assay kits of glutathione (GSH), superoxide dismutase (SOD) and glutathione peroxidase (GPx) were bought from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The antibodies against β-actin, Lamin B, NQO1 and Nrf2 were purchased from Proteintech group, Inc. (Wuhan, China). The antibodies against JNK, p-JNK, Akt and p-Akt were purchased from Biosynthesis Biotechnology Co., Ltd. (Beijing, China). The antibodies against p38 and p-p38 were purchased from Ruiying Biological Technology co., Ltd. (Suzhou, China). The antibodies against ERK and p-ERK were from Cell Signaling Technology (Boston, MA, USA). The nuclear/cytosol fractionation kit was purchased from Sangon Biotech Co., Ltd. (Shanghai, China). LY294002 (an Akt inhibitor), SP600125 (a JNK inhibitor), SB203580 (a p38 inhibitor) and U0126 (an ERK inhibitor) were obtained from Selleck Chemicals (Shanghai, China).

2.2. Origin and maintenance of parental zebrafish

Adult zebrafish were obtained from commercial dealer (Jiangbei Sanhe aquarium, Chongqing, China). 10 fish were kept in a 1.2 L acrylic tank under the following conditions: 28.5 ± 1 °C, with a 14/10 h light/dark cycle. Fish were fed three times a day and the feed was purchased from Tai Port Technology co., Ltd. The night before, 1 female and 2 males be separated in the same tank to breed. In the next morning, Embryos were obtained from natural spawning that was stimulated by turning on the light. Collection of embryos and picking out those being in good development status were completed within 2 h.

2.3. Waterborne exposure of embryos to COP and AAPH

All the procedures were conducted according to the previously reported methods with few modifications [26]. The embryos (n = 8) were transferred to individual wells of 24-well plates containing 1 mL embryo media (0.25 mg/mL sea crystal solution filled with oxygen for a night). From approximately 6–7 h post-fertilisation (6–7 hpf), except for normal and model groups, positive control (Vit. C) and three therapy groups (COP) were respectively given Vit. C and COP to make sure the final concentration of Vit. C was 2.5 μg/mL with reference to the dosage from literature [27] and the low, medium and high dosages of COP was 2.5, 5 and10 μg/mL. After the incubation until 12 hpf, the embryos of model group were treated with 12.5 mM AAPH and the drug groups were co-treated with 12.5 mM AAPH and corresponding drugs (Vit. C and COP) for up to 24–hpf. Then, embryos were rinsed using fresh embryo media. Subsequently, the normal and model groups were maintained in embryo media and the rest of groups were of embryo media containing Vit. C or COP. Till 2 dpf, after embryos were stopped being treated with drugs and fresh embryo media was replaced, all the indicators were tested and the total RNA was extracted because zebrafish larvae typically hatch within 2–3 dpf at 28.5 °C [28].

2.4. Detection of AAPH-induced intracellular ROS generation, heart beating rate, lipid peroxidation and cell death in zebrafish embryos

ROS production in zebrafish embryos was analyzed through using DCFH-DA, an oxidation-sensitive fluorescent probe dye. DCFH-DA was deacetylated by nonspecific esterase when entering into the cells, which was further oxidised to the highly fluorescent compound dichlorofluorescein (DCF) in the presence of cellular ROS [29]. Lipid peroxidation was measured to assess the membrane damage degree in zebrafish model. 1, 3-Bis (diphenyl-phosphino)-propane (DPPP) is a fluorescent probe for monitoring lipid peroxidation in cell membrane and DPPP is non-fluorescent, while DPPP oxide is fluorescent [30]. Cell death was tested in live zebrafish embryos using acridine orange staining [31], a nucleic acid selective metachromatic dye that can interact with DNA and RNA by intercalation or electrostatic attractions. Acridine orange stains cells when plasma membrane permeability was disturbed. So, it preferentially stains necrotic or very late apoptotic cells.

The heart beating rate of both atrium and ventricle was tested on 2 dpf. Recording of atrial and ventricular contractions was performed for 1 min under the microscope, and then we counted it to get the heart beating rate. Also on 2dpf, the embryos were treated with DCFH-DA solution (20 μg/mL), DPPP solution (25 μg/mL) or AO solution (7 μg/mL) for 1 h, 1 h and 30 min in the dark at 28.5 ± 1 °C, respectively. After incubation, the embryos were rinsed with fresh embryo medium and anaesthetized by ethyl 3-amino-benzoate methanesulfonate salt (0.2 mg/mL) before observation. The images of stained embryos were photographed under the fluorescence microscope, which was equipped with a DS-U3 digital camera (Tokyo, Japan). A fluorescence intensity of individual