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Original article

Procyanidins from *Nelumbo nucifera* Gaertn. Seedpod induce autophagy mediated by reactive oxygen species generation in human hepatoma G2 cells

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ABSTRACT

In this study, autophagic effect of procyanidins from lotus (*Nelumbo nucifera* Gaertn.) seedpod (LSPCs) on human hepatoma G2 (HepG2) cells, and the inherent correlation between autophagic levels and reactive oxygen species (ROS) generation were investigated. The results showed that LSPCs increased monodansylcadaverine (MDC) fluorescence intensity and LC3-I/LC3-II conversion in HepG2 cells. In addition, the typically autophagic characteristics (autophagosomes and autolysosomes) were observed in LSPCs-treated cells, but not found in the cells treated with autophagy inhibitor 3-methyladenine (3-MA). Furthermore, the elevated ROS level was in line with the increasing of autophagy activation caused by LSPCs, however, both 3-MA and the ROS scavenger *N*-acetylcyteine (NAC) inhibitors effectively suppressed the autophagy and ROS generation triggered by LSPCs. As a result, these results indicated that LSPCs induced HepG2 cell autophagy in a time- and dose-dependent manner, and promoted reactive oxygen species (ROS) generation on HepG2 cells. Moreover, we found that LSPCs caused DNA damage, S phase arrest and the decrement of mitochondria membrane potential (MMP) which were associated with ROS generation. In summary, our findings demonstrated that the LSPCs-induced autophagy and autophagic cell death were triggered by the ROS generation in HepG2 cells, which might be associated with ROS generation through the mitochondria-dependent signaling way.

1. Introduction

Autophagy refers to a process in which cellular organelles and macromolecules are degraded for recycling of bioenergetic components [1]. The autophagic process includes a series of steps, including initiation, elongation and expansion of the phagophore assembly site (PAS), phagophore, formation and maturation of double-membrane vesicle termed autophagosome, and autophagosomes subsequently fuse with lysosomes to form autolysosomes for degradation [2]. In early stages of cancer development, quality control of autophagy suppresses tumor growth and exerts its anti-carcinogenic function by preventing metabolic or oxidative stress, maintaining normal mitochondrial function and safeguarding against DNA damage [3]. In the late stage of oncogenesis or established tumors, basal autophagy ensures cellular homeostasis by preventing waste accumulation and removing damaged and old organelles. However, unrestrained autophagy could potentially result in progressive consumption of cellular components and subsequently induce cancer cell death [4–6].

Emerging evidence indicates oxidative stress plays an important role in the tumor development. Many conventional anti-cancer drugs, including vinblastine, doxorubicin, camptothecin, cisplatin and inostamycin, exhibit anti-tumour activity via ROS generation [7]. Reactive oxygen species (ROS) have been implicated as a signal for general autophagy. Accumulation of ROS within the mitochondria leads to a collapse of mitochondrial membrane potential and a transient increase in ROS generation [8]. Autophagy can act as a ROS scavenger, maintaining genomic integrity, and prevents tumorigenesis [8,9]. However, high levels of ROS can oxidize cell constituents and promote autophagy [10]. Studies have shown that ursolic acid induces autophagy in U87MG cells via ROS-dependent endoplasmic reticulum stress [11]. However, to fully reveal the complex paradoxical role of autophagy and ROS in

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cancer development as well as in cancer therapy, remain further research.

Hepatocellular carcinoma (HCC) is the third most deadliest and fifth most common cancer across the world. Each year more than 500,000 new patients are diagnosed with HCC in the world [12]. HCC is a deadly disease with very poor prognosis and characterized by rapid cell proliferation and strong expression of antiapoptotic genes, suggesting that HCC is mainly due to incomplete cell-cycle arrest and apoptosis-resistance under conventional therapies [13–15]. Traditional chemotherapeutic drugs in the treatment of HCC tend to cause severe toxicity to normal tissues at high concentrations, such as adriamycin, doxycycline. Recently considerable emphasis confirmed the importance of the naturally available botanicals and indicated that these botanicals can be useful as a chemopreventive or chemotherapeutic agent for certain diseases, including cancers [16]. A mass of dietary flavonoids or polyphenolic substances, have been reported to possess substantial anti-carcinogenic and antimutagenic activities [16], such as proanthocyanidins. Grape seed proanthocyanidins (GSPs) are promising bioactive phytochemicals that have shown anti-carcinogenic effects in skin, prostate, breast and colorectal cancer models without apparent toxicity in vivo [17,18].

Epidemiological studies suggest flavonoids, polyphenolic substances, proanthocyanidins, compounds naturally present in many foods, may inhibit the proliferation of various cancer cells including the breast, colon, lung, prostate, and pancreas [19]. Our previous works have shown that chestnut shell procyanidians (CSPCs) with different concentrations of CSPCs have different effects on HepG2 cells. In low concentrations, CSPCs acts as a ROS scavenger can eliminate oxygen free radicals and maintain cellular homeostasis [20]. However, high concentration of CSPCs leads to ROS accumulation, inducing apoptosis in HepG2 [21]. In addition, we demonstrated that CSPCs could induce autophagy and autophagic cell death which mediated by ROS [20]. These all show the complexity of the tumor cell development, and the relationship between autophagy and ROS has not been fully elucidated and required further study. Lotus seedpod belongs to mature torus of *Nelumbo nucifera* Gaertn., which contains rich procyanidians. Most lotus seedpod had been abandoned in processing process, except when sometimes used as a traditional medicine with hemostasis function and for eliminating bruise. Procyanidins from lotus seedpod (LSPCs) are constituted by a variable number of flavan-3-ols units linked together through C4–C8 (or C6) interflavanoid bonds, and the oligomeric procyanidins are considered to be the main active constituents of LSPCs [22]. It is known that malignant tumors are generally considered as defective apoptosis. Therefore, it has become a new thought in current oncotherapy by using autophagy inducers to trigger excessive autophagy and induce autophagic cell death. Raina et al. indicate that grape seed extract is effective in killing tumor cells via ROS-triggered autophagic pathway [23]. LSPCs and CSPCs have similar chemical composition and biological effects, such as antioxidant activity, anti-cancer activity, radiation resistance, etc. However, it is unknown that whether LSPCs have the same effects on HepG2 and the mechanisms between autophagy and ROS induced by LSPCs. Therefore the present study is performed to investigate the role of LSPCs contribution to autophagy and ROS production.

2. Materials and methods

2.1. Cells and reagents

Cell line: HepG2 cells were purchased from the Institute of Biochemistry and Cell Biology (SIBS, CAS; Beijing, China). HepG2 cells were grown at 37 °C, 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (V/V) heat-inactivated newborn calf serum, 100 U/mL penicillin and100 μg/mL streptomycin. Cells were harvested by trypsinization (0.5% trypsin/2.6 mM EDTA), and washed with phosphate buffered solution (PBS).

Reagents: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tertazolium bromide (MTT), 3-methyladenine (3-MA), *N*-acetylcysteine (Nac), monodansylcadaverine (MDC), antibodies against LC3 and rapamycin were purchased from Sigma Chemical Corp. (St. Louis, MO, USA). DMSO were obtained from AMRES CO. (USA). Antibodies against β-actin were provided by Abcam (Cambridge, UK). 5,5′,6,6′-tetrachloro-1,1,3′,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1,Molecular Probe), 2′,7′-dichlorofluorescein diacetate (DCFH-DA), ribonuclease (RNase), propidium iodide (PI) were purchased from Beyotime Institute of Biotechnology (Nantong, China).

2.2. Preparation of LSPCs

Lotus (*N. nucifera Gaertn.*) seedpod was collected from Honghu Lantian Lake (Hubei, China), named No. 2 Wuhan and authenticated by Wuhan Plant Institute, Chinese Academy of Science.

LSPCs was extracted, purified, and characterized by the method described previously [22]. Briefly, the lotus seedpod was extracted three times with acetone/water (V/V, 7:3). Then the acetone–water extract was purified by Sephadex LH-20 column chromatography, with a purity of >98%, LSPCs. The main molecular weight distribution of LSPCs was confirmed to be in the range 291.1–1155.3, and the LSPCs polymerization was ≤4. ESI-MS analysis indicates that the extract contains monomers, dimers, and tetramers of proanthocyanidins, in which the amounts of dimers are greatest, and catechin and epicatechin are the base units [24,25].

To avoid batch-to-batch and/or lot-to lot variation in LSPCs preparation and to maintain consistency of our studies, we prepared a large quantity of one batch/lot of LSPCs for all our studies.

2.3. Cell viability assay

HepG2 cells were plated in a 96-multiwell plate at a density of 2×10^4 cells/well. After 24 h incubation, cells were treated with increased concentrations of LSPCs (0, 12.5, 50, 100, 200 and 400 ig/mL) for 6, 12, 24, 36, 48, 60, 72 and 96 h. Cell viability was then assessed using the MTT colorimetric assay. Briefly, the cells received the 100 μL MTT solution (1 mg/mL of MTT dissolved in DMEM) and were incubated in the dark at 37 °C for 4 h; then the MTT solution was carefully removed and the precipitated formazan were dissolved in 150 μL DMSO. Absorbance (A) optical density (OD) of each well was determined by microplate reader (Multiskan MK3, USA) at 490 nm of wavelength. Each time point was repeated five times and the mean and standard errors were calculated. Calculation formula of the cellular growth inhibiting ratio is: inhibition ratio = (1-average OD value of experiment group/average OD value of control group) × 100%. The IC50 was defined as the concentration of LSPCs that reduced cell viability by 50% and was calculated by the logit method.

2.4. MDC staining

Monodansylcadaverine (MDC) was employed to stain autophagosomes. About 1×10^5/well HepG2 cells were respectively seeded into 6-well plates for 24 h and treated with indicated concentrations of LSPCs and time periods or 1 nmol/L rapamycin for 24 h, or the cells were pre-treated with 1 mM 3-MA for 1 h and co-incubated with LSPCs for 24 h. Then the cells were incubated with