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Original article

Oleuropein protects L-02 cells against H_2O_2-induced oxidative stress by increasing SOD1, GPx1 and CAT expression

Ce Shi[a], Xiangrong Chen[a], Zuojia Liu[b], Rizeng Meng[c], Xingchen Zhao[a], Zonghui Liu[a], Na Guo[a,*]

[a] Department of Food Quality and Safety, College of Food Science and Engineering, Jilin University, 130062 Changchun, PR China
[b] State Key Laboratory of Electroanalytical Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, 130022, Changchun, PR China
[c] Jilin Entry-Exit Inspection and Quarantine Bureau, 130062, Changchun, PR China

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ABSTRACT

Oleuropein (OL), a natural phenolic compound, comprises the major constituent of *Olea europea* leaves and unprocessed olives, and OL is considered to be the principal components that confer the characteristic taste and stability of olive oil. Oxidative damage induced by H_2O_2 treatment can irreversibly damage the L-02 cells, resulting in cell death and apoptosis. Whether the effects of oxidative stress could be attenuated in cultured human L-02 cells by incubation with OL is still unknown. In this research, the function of OL in protecting human L-02 cells against H_2O_2 induced cell death and cell apoptosis was investigated, and the mechanism by which OL underlies the effect was also examed. L-02 cells were exposed to 100 μM H_2O_2 with or without OL pretreatment at different concentrations. Cell viabilities were monitored by WST-1 assay. ALT, AST and LDH production in culture medium were also determined. ROS levels were detected by L-012 chemiluminescence, and OL increased SOD1, CAT and GPx1 expression in a concentration-dependent manner. Further studies showed that OL also inhibited H_2O_2-induced P38 and JNK phosphorylation but enhanced ERK1/2 phosphorylation in a dose-dependent manner. These findings suggested that OL as a potent antioxidant agent and a natural compound found in several plants, may be exploited as a potentially useful method for hepatopathy prevention.

1. Introduction

The olive, *Olea europaea*, is an evergreen tree that grows in the Mediterranean, Asia and Africa [1]. Its fruit is commonly used as a source of olive oil, and olive leaves are also known to be a natural resource of various beneficial polyphenols, which are commonly used as traditional medicines for malaria and fever in Mediterranean countries. Olive leaves are considered as a cheap raw material which can be used as a useful source of high-added value products (phenolic compounds) [2]. In addition, *Olea europaea* extract inhibits proliferation in a variety of cancer cell lines, such as human adenocarcinoma cells HT115, human glioblastoma cells T98G, and human leukemia cells HL-60. The main constituent of the leaves and unprocessed olive drupes of *Olea europaea* is oleuropein (OL), which is deemed to have great potential as an antioxidant and food additive, but also as a possible therapeutic tool [3]. Recently, more scientific interests have been focused on the association between the olive phenols and human health. OL is known to possess several positive biological effects for human health, including antioxidant, antiinflammatory, antiobesity, antivira, neuroprotective and antimicrobial activities. Notably, recent studies have shown that OL inhibits growth and induces apoptosis in prostate cancer cell lines LNCaP, DU145, human lung carcinoma A549 cells and human breast cancer cells MCF-7 [4]. Thus, this natural product has attracted significant scientific and research interest, mainly connected with its potential protective role against infections and diseases, as well as the risk of developing breast, prostate and colon cancers, cardiovascular diseases and diabetes.

Reactive oxygen species (ROS), a group of chemically reactive molecules derived from oxygen are generated to some extent in most of mammalian cells under normal conditions due to partial reduction of molecular oxygen in mitochondria [5,6]. Excessive ROS can cause oxidative damage to attack lipid membranes, proteins and deoxynucleic acids [7], and eventually contribute to cells injured or died. And the antioxidant defense is confirmed to protect the body from a normal extent of ROS injuries. Thus,

* Corresponding author.
E-mail address: abas1980@163.com (N. Guo).

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inhibition of free radical-induced injury may provide a feasible approach to prevent hepatocytes from excessive exposure to oxidative stress. To mitigate and repair the damage caused by ROS, cells have developed a complex antioxidant response, which usually encompasses varieties of small molecules and some well-characterized enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx).

Oxidative stress, which is a physiological status whereby intracellular free radicals exceed the antioxidant abilities, has been recognized as a key factor in the pathogenesis of several chronic liver diseases, such as hepatitis, alcoholic and non-alcoholic fatty liver diseases [6,8]. H_2O_2, a typical oxidant, can result in oxidative stress and induce tissues injuries even the apoptosis in cells [9]. Therefore, the objective of the present study was to investigate the potential protective effect and mechanism of OL against oxidative stress induced by H_2O_2 in human liver L-02 cells, and whether OL could reduce H_2O_2-induced cell apoptosis and cell death in cultured human L-02 cells was also examined.

2. Materials and methods

2.1. Chemical reagents and cells culture

OL was purchased from Chengdu Must Bio-Technology Co., Ltd. (Chengdu, China). The immortalized normal human liver cell line L-02 was obtained from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The L-02 cells line is a human normal hepatic cells line, so the human hepatocyte L-02 cell line was selected to establish an in vitro model to evaluate the protective effect of OL against H_2O_2-induced liver cell damage. Dulbecco's modified Eagle's medium (DMEM) was obtained from Corning (New York, USA) and fetal bovine serum (FBS) was obtained from Invitrogen-Gibco (New York, USA). The L-02 cells were cultured in DMEM supplemented with heat-inactivated (56 °C, 0.5 h) 10% FBS at 37 °C in a humidified atmosphere of 5% CO_2. 4-[3-[4-iodophenyl]-2-4(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulfonate] (WST-1) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma-Aldrich (Saint Louis, USA). Anti-c-Jun N-terminal kinase (JNK), anti-phosphorylation of JNK (p-JNK), anti-P38, anti-phosphorylation of P38 (p-P38), anti-extracellular-regulated kinase 1/2 (ERK1/2), anti-phosphorylation of ERK1/2 (p-ERK1/2) were purchased from Cell Signaling Technology (Boston, USA), anti-superoxide dismutase 1 (SOD1), CAT and anti-glutathione peroxidase 1 (GPx1) were purchased from Abcam (Cambridge, UK). Diagnostic kits for alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) were provided by Sigma-Aldrich (Saint Louis, USA).

2.2. Radical-scavenging activity

The antioxidant activity of OL evaluated by DPPH radical scavenging activity was performed according to previous study [10], and evaluated by an ELX800 microplate reader (Bio-Tek, USA). Ascorbic acid (AA) was used as a reference material. DPPH radical scavenging activity (*SA* %) was calculated as follows:

$$SA\ \% = [(A_{DPPH} - A_S)/A_{DPPH}] \times 100$$

where A_S is the absorbance of the solution containing the sample at 517 nm, A_{DPPH} is the absorbance of the DPPH solution. The concentration for 50% of maximal effect (EC_{50}) values, which was calculated from the linear regression algorithm of the graph plotted inhibition percentage were used to express the results. Lower EC_{50} values mean greater antioxidant activity.

2.3. Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of OL was measured on the basis of previous studies with slight modifications [11,12]. In this assay, the reaction mixture consist of 0.1 ml ethylene diamine tetraacetic acid, 0.01 ml of $FeCl_3$, 0.01 ml of H_2O_2, 0.36 ml of deoxyribose, 1 ml OL solution (20 μM–2560 μM), 0.33 ml phosphate buffer saline buffer and 0.1 ml AA. Then the mixture was incubated for 2 h at 37 °C. Finally, a 1 ml portion of the mixture was used to mix with 10% trichloroacetic acid (1 ml) and 0.5% thiobarbituric acid (1 ml) to develop the pink chromogen. The optical density was measured at 532 nm. AA was used as reference standard for this assay. The hydroxyl radical scavenging activity (*SA* %) of OL was calculated as follows:

$$SA\ \% = [(A_{control} - A_{samples})/A_{control}] \times 100$$

where $A_{control}$ is the absorbance of control at 532 nm, $A_{samples}$ is the absorbance of OL samples at 532 nm.

2.4. Cell viability assay

The proliferation of L-02 cells was evaluated using a sulfonated tetrazolium salt WST-1. The measurement is based on the ability of viable cells to cleave tetrazolium salts by mitochondrial dehydrogenases. In this assay, the cytotoxicity of H_2O_2, maximal non-cytotoxic concentration of OL and the cytotoxicity of both H_2O_2 and OL treatment were measured respectively. Briefly, L-02 cells were plated at a density of 1×10^4 cells/well in 96-well microplates in DMEM containing 15% FBS and incubated at 37 °C in a humidified atmosphere of 5% CO_2. After a 24 h incubation, at approximately 80% confluence, the assays were performed respectively as follows: (1) Cells were incubated with OL and H_2O_2 respectively at various concentrations for 12 h. (2) Cells were pretreated with 7.4 μM, 14.8 μM and 29.6 μM OL for 12 h. After incubation for the indicated time, cells were treated with 100 μM H_2O_2 in combination for 24 h. To assay for cell viability, 10 μl/well WST-1 reagent was added and incubated for 2 h at 37 °C and 5% CO_2. The absorbance of the samples was measured at 450 nm by using a microplate reader (Bio-Tek, USA).

2.5. Determination of ALT, AST and LDH activities

The activities of ALT, AST and LDH, which were used as the biochemical markers for the early acute hepatic damage, were measured on the basis of the methods described by previous studies [13–15] using commercially available assay kits according to the manufacturer's instructions. In brief, L-02 cells were plated at a density of 2×10^6 cells/dish in a 100 mm-diameter sterile culture dish in DMEM containing 15% FBS and incubated at 37 °C in a humidified atmosphere of 5% CO_2. After 24 h incubation, cells were pretreated with or without OL at different concentrations (7.4, 14.8 and 29.6 μM) for 4 h, cells treated without OL served as control groups. And then the cells were stimulated with 100 μM H_2O_2 for a further 4 h. Finally, the cell culture medium was harvested and centrifuged at 1800 × *g* for 10 min at 4 °C to remove floating cell, and the supernatant was collected for enzyme activity assay. The enzymatic activities were expressed as unit per liter (U/L).

2.6. Western blot for SOD1, GPx1 and CAT protein analyses

The L-02 cells (1×10^6 cells) were seeded in a 100 mm plate and cultured overnight. Then the cells were exposed to OL with different concentrations of 7.4 μM, 14.8 μM and 29.6 μM for 24 h respectively, and no OL treatment served as a control. Cellular