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Protective effects of the citrus flavanones to PC12 cells against cytotoxicity induced by hydrogen peroxide

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ABSTRACT

Oxidative stress has been considered as a major cause of cellular injuries in a variety of clinical abnormalities. One of the plausible ways to prevent the reactive oxygen species (ROS)-mediated cellular injury is dietary or pharmaceutical augmentation of endogenous antioxidant defense capacity. In this study, we investigated the protective actions of citrus flavanones naringin and nobiletin against the cytotoxicity induced by exposure to hydrogen peroxide (H_2O_2) (150 μM, 3 h) in PC12 cells. The results showed that naringin and nobiletin inhibited the decrease of cell viability (MTT reduction), prevented membrane damage (LDH release), scavenged ROS formation, reduced caspase-3 activity, and attenuated the decrease of mitochondrial membrane potential (MMP), respectively, in H_2O_2-induced PC12 cells. Meanwhile, naringin and nobiletin increased superoxide dismutase (SOD) and glutathione (GSH) activity, while decreased malondialdehyde (MDA), the production of lipid peroxidation, in H_2O_2-induced PC12 cells. In addition, the percentage of cells undergoing H_2O_2-induced apoptosis was decreased in the presence of naringin and nobiletin. These results first demonstrate that naringin and nobiletin, even at physiological concentrations, have neuroprotective effects against H_2O_2-induced cytotoxicity in PC12 cells. All the above results suggest that these dietary antioxidants are potential candidates for use in the intervention for neurodegenerative diseases.

For organisms living in an aerobic environment, exposure to reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide anion, and hydroxyl radical, is unavoidable. However, the brain is very sensitive to oxidative stress, because it has low-to-moderate activity with respect to enzymatic defense systems, compared with other organs [5]. Oxidative damage in brain occurs in most neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, and Huntington's disease [1]. As a major ROS, H_2O_2 causes lipid peroxidation and DNA damage in cells. H_2O_2 is generated during amyloid aggregation, dopamine oxidation, and brain ischemia/reperfusion [15]. Thus, formed H_2O_2 is readily converted into highly toxic hydroxyl radical by Fenton chemistry and further damages lipids, proteins, and DNA. This oxidative damage could lead to mitochondrial dysfunction, calcium imbalance, and apoptosis in neuronal cells [6].

The rat pheochromocytoma line PC12 provides a useful model system for neurological and neurochemical studies [19]. In PC12 cells, oxidative stress induces apoptosis rather than necrosis [9]. Increased production of ROS or a poor antioxidant defense mechanism leads to physiological dysfunction and progressive damage in the cells. Recent studies showed that H_2O_2 induces cytotoxicity on PC12 cells including membrane and nuclear damage, decrease in mitochondrial membrane potential (MMP) and antioxidant enzyme activities, such as superoxide dismutase (SOD), accumulation of caspase-3 activation, increase in ROS level, and depletion of glutathione (GSH) [8].

In an effort to prevent or diminish ROS-induced damage, investigators have evaluated compounds that prevented their generation and reduced neuronal damage. In recent years, studies showed that food-derived antioxidants, such as vitamins and phytochemicals, have received growing attention for their function as chemopreventive agents against oxidative damages [2]. Therefore, natural flavonoids with more of a lipophilic chemical structure and antioxidant properties are promising candidates for neurodegenerative intervention [9]. Citrus flavonoids, mainly existing in the pericarp of citrus fruits, have been attracting peoples' attention for decades due to their versatile bioactivities and high consumption by consumers. Citrus flavonoids are generally categorized into two groups, flavanone glycosides (naringin, hesperidin, neo-hesperidin, etc.) and polymethoxylated flavones (nobiletin, sinensetin, tangeretin, etc.). Among them, naringin and nobiletin (Fig. 1) are the abundant flavonoids and are found mainly in oranges, tangerines,

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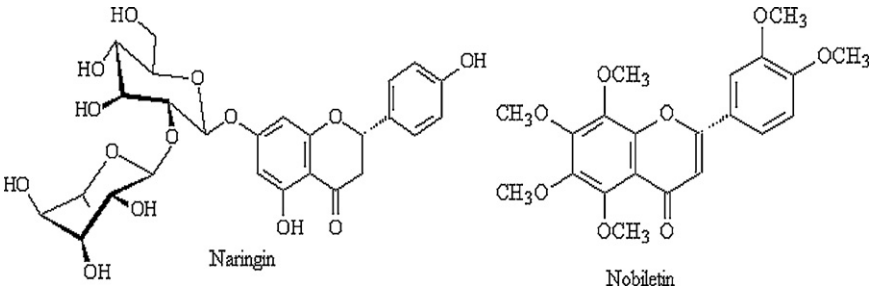

Fig. 1. Chemical structures of naringin and nobiletin.

lemons, and grapefruits. Naringin and nobiletin were known to exhibit free-radical scavenging activity [16]. Further, naringin was proved to possess antioxidative activity in high cholesterol-fed rabbits through improving the plasma lipid levels and increasing the plasma antioxidant activity [12,13]. Moreover, naringin can protect mouse liver and intestine against the radiation-induced damage by elevating the antioxidant status and reducing the lipid peroxidation [11]. However, the intracellular ROS scavenging activities of these flavanones have not been tested. We think that naringin and nobiletin show potential for use in neurodegenerative intervention and thus the aim of this study is to investigate the protective effects of naringin and nobiletin on PC12 cells against cytotoxicity induced by H_2O_2 *in vitro*.

PC12 cell line was purchased from Cell Bank of Chinese Academic of Science and cultured in DMEM (Gibco) with 10% calf serum (Hangzhou Sijiqing Co., China), 10 mmol/L HEPES, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cultures were kept at 37 °C in a humidified atmosphere incubator with 5% CO_2/95% air. The medium was changed every other day. Naringin and nobiletin were freshly prepared as stock solution in dimethylsulfoxide (DMSO) and diluted with DMEM before the experiment. 0.1% (v/v) DMSO had no protective or toxic effects by itself. Control group was performed in the presence of 0.1% (v/v) DMSO under the same culture conditions. In all experiments, PC12 cells were placed within 96-well culture plates or 6-well culture plates (10^4 cells/well or 10^6 cells/well), 12 h later, cells were preincubated with indicated concentrations of citrus flavanones or Vitamin E (10 μM) for 30 min and later H_2O_2 (150 μM) was added to the medium for 3 h. The citrus flavanones were not removed after the addition of H_2O_2.

Cell survival was evaluated by two different methods: morphological observation with phase-contrast microscope (Nikon) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay. In the MTT assay, cells were exposed to indicated concentrations of naringin, nobiletin, Vitamin E and H_2O_2 for designated times frames, then the supernatant was discarded and MTT (Sigma) solution (final concentration, 0.5 mg/mL) was added and further incubated for 4 h at 37 °C, and then were replaced with DMSO to solubilize the precipitated dye. Absorbance was measured on an Automated Microplate Reader (Bio-Rad 550) at 570 nm.

The plasma membrane damage of PC12 cells was determined by the release of LDH into medium. PC12 cells in 6-well plates were cultured and treated according to the procedures described above. The medium was harvested for the spectrophotometrical determination of the amount of LDH release using an assay kit (Nanjing Jiancheng Co., China) according to the manufacturer's protocol, and the absorbance of samples was read at 490 nm.

For assays of lipid peroxide and antioxidant substances, after the treatment, cells were harvested by centrifugation and washed with PBS (pH 7.0) for twice, and then re-suspended in physiological saline solution. The cells were lysed by sonication, and then the suspension was used to measure the levels of malondialdehyde (MDA), GSH, and SOD according to the manufacturer's instructions (Nanjing Jiancheng Co.).

The mitochondrial membrane potential (MMP) was evaluated using the fluorescent rhodamine dye, Rh123 [14]. It is a cell permeable cationic dye that preferentially partitions into mitochondria based on the highly negative MMP. At the end of the experiment, about 1×10^6 cells were harvested by centrifugation. After washed twice with PBS, the cells were incubated with Rh 123 (Sigma) (10 μM) for 30 min at 37 °C in the dark. Then cells were washed and suspended in PBS. The MMP was analyzed by FACScan flow cytometer (Becton Dickinson, USA) at an excitation wavelength of 480 nm and an emission wavelength of 530 nm.

The production of intracellular ROS was quantified by using a DCFH-DA assay [17]. Cell-permeant nonfluorescent DCFH-DA has been shown to be oxidized to the high fluorescent 2,7-dichlorofluorescin (DCF), which is retained within the cells, in the presence of ROS. The cells on 6-well plates were incubated with 10 μM DCFH-DA (Sigma) in the DMEM cultured for 30 min. Then after the compounds treatment, cells were harvested and suspended in PBS. The fluorescence intensity was measured by FACScan flow cytometer at an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Extent of apoptosis was measured by Annexin V-FITC/propidium iodide (PI) apoptosis detection kit (Invitrogen. USA). Briefly, at the end of the treatment, cells were harvested and washed twice with PBS and then the cells were used for assay according to the manufacturer's instructions. After that, it was analyzed by FACScan flow cytometer [18]. The fraction of cell population in different quadrants was analyzed using quadrant statistics.

Caspase-3 activity was determined using a caspase-3 activity detection assay kit (KeyGEN, Nanjing). Briefly, after cell lysis and centrifugation at 10 000 × *g* for 10 min at 4 °C, the supernatant from lysed cells was added to the reaction mixture containing dithiothreitol and caspase-3 substrate (N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide) and further incubated for 1 h at 37 °C. Absorbance was measured on an Automated Microplate Reader (Bio-Rad 550) at 405 nm.

All the assays were carried out at least in three experiments with fourfold sample. The results were expressed as means ± S.D. and were analyzed by pair *t*-test.

As shown in Fig. 2B, after cells exposed to 150 μM H_2O_2 for 3 h, PC12 cells exhibited a marked decrease in cell number and most cells demonstrated cell shrinkage and membrane blebbing, which are normally associated with the occurrence of apoptotic cell death. Meanwhile, cell viability was decreased ($P < 0.01$) to 47.1% of control group (Fig. 2F), suggesting that PC12 cells were very sensitive to H_2O_2-induced cell injury. However, pretreatment of cells with naringin, nobiletin and Vitamin E significantly ($P < 0.01$) elevated the cell viability to a range of 72.3–89.1%, and this pretreatment also mitigated such morphological features of H_2O_2-induced PC12 cells (Fig. 2C–E). Moreover, the citrus flavanones at these concen-