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Synthesis of glutathione C_{60} derivative and its protective effect on hydrogen peroxide-induced apoptosis in rat pheochromocytoma cells

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Abstract

Oxidized glutathione C_{60} derivative has been synthesized and characterized in our research. As a novel derivative of C_{60}, the glutathione C_{60} derivative is soluble in dimethylsulfoxide, dimethylformamide and dimethylacetamide. Rat pheochromocytoma (PC12) cells are treated with hydrogen peroxide and underwent cytotoxicity; apoptotic death is determined by MTT assay, flow cytometry analysis, PI/Hoechst 33342 staining and glutathione assay. The results suggest that glutathione C_{60} derivative has the potential to prevent oxidative stress-induced cell death without evident toxicity.

Keywords: Glutathione C_{60} derivative; Hydrogen peroxide; Apoptosis; PC12 cells

There is ample evidence to suggest that fullerene and its derivatives possess properties which hint at their use in biomedicine, such as DNA photocleavage [2,15], neuroprotection [5,6], and inhibition of apoptosis [1,14]. In recent years, covalent modification of C_{60} with water-soluble groups has been of significant interest. The modification strategy is not only an effective way to solubilization of C_{60} but also particularly important to the preparation of new compound classes with unprecedented properties.

Glutathione is a very interesting, very small molecule that is produced by the body and found in every cell. Because glutathione exists within the cells, it is in a prime position to neutralize free radicals. It has potential to fight almost any disease, particularly those associated with aging, since free radical damage is the cause of many of the diseases of old age. It also has potentially widespread health benefits because it can be found in all types of cells, including the cells of the neural system [12,18]. Similarly, previous results have shown that fullerene derivatives have excellent ability to protect cells against apoptosis induced by free radicals [4,10,11]. Thus, these two molecules share some important properties; the opportunity to combine C_{60} and glutathione appears as a desirable way to develop a new molecule provided with properties that are inherent in both components.

As the base of further studies aim for potential applications in neural protection and anti-apoptosis, it is urgent to create functional C_{60} with glutathione. Glutathione contains multiple amino and carboxyl groups which offer flexible and efficient routes, which make the modification easier and more effective. Furthermore, modified C_{60} by glutathione is also an effective way to the solubilization of C_{60}. In this paper, we report the first successful covalent modification of C_{60} with a water-soluble oxidized glutathione (GSSH). We thought it worthwhile to analyze the possible protective role and mechanism of the oxidized glutathione C_{60} derivative (OGFD) against oxidative stress and apoptosis induced by hydrogen peroxide in cultured PC12 cells (Fig. 1).

Oxidized glutathione (1 mmol) and tetrabutylammonium bromide (0.5 g) were dissolved in 3 mL water, and then 10 drops of alkaline bromine water was added, the resultant solution was added to a C_{60} toluene solution (0.2 mmol, 90 mL) under stirring. The solution was stirred at room temperature under nitrogen atmosphere. The solution was stirred for 48 h to make sure the reaction was complete. The resultant mixture was filtered through a 0.22 μm pore size nylon membrane. A black residue

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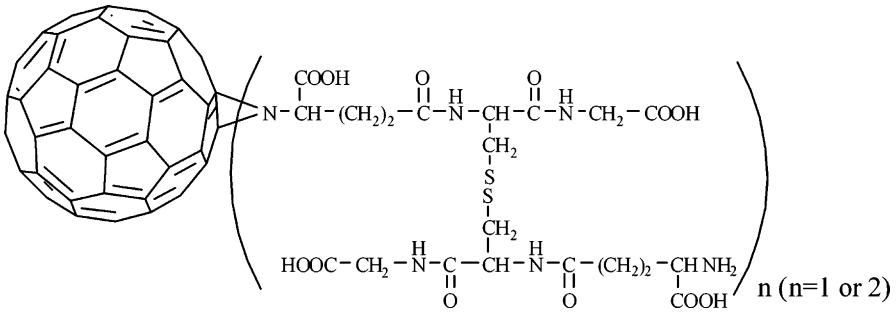

Fig. 1. Schematic representation for the chemical structure of oxidized glutathione C_{60} derivative.

was obtained. The residue was sonicated in water (500 mL) for 1 h, filtered, washed with another 500 mL of water, and then repeatedly extracted with water in a Soxhlet apparatus for 24 h. The final product was dried at 40 °C under vacuum for 24 h.

Analytic data of the synthesized and purified compound are shown below. IR(KBr) ν: 3381, 1710, 1601, 1260, 705, 527, 548, 572 (C_{60} core), cm^{-1}; ^1H NMR(D_2O) δ (ppm): 7.14(m), 7.23(m), 4.33(s), 3.26(s), 2.71(d), 2.32(d), 1.57(s), 1.38(d), 1.16(s), 0.95(s); ^{13}C NMR(D_2O) δ (ppm): 14.2, 19.7, 23.7, 29.5, 56.5, 58.2, 139.8–150.8, 181.4–187.8; ESI-MS *m/z* (%): 1940.3 (M^+, 100). The reaction on fullerene gives mono-adduct and bis-adducts demonstrated by mass spectroscopy. That means glutathione fullerene derivative was obtained by a mixture of mono-adduct and bis-adducts.

PC12 cells were maintained in DMEM supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum at 37 °C. All cells were cultured in poly-D-lysine coated culture dishes. After 48 h incubation, cells were switched into serum-free medium for treatment. The OGFD dissolved in dimethylsulfoxide and then added to the plates, followed by incubation for 1 h at 37 °C. Hydrogen peroxide solution was then added to the plates for 24 h at 37 °C. The final concentration of dimethylsulfoxide in media is 27.5 μg/mL.

The fluorescent probe 2,7-dichlorofluorescein diacetate (DCF-DA) was used to monitor the intracellular accumulation of ROS [13]. Cells (1×10^6 cells per 3 mL in 6-well plates) were rinsed with D-Hanks solution and 10 μM DCF-DA was loaded. After 20 min incubation at 37 °C, the cells were harvested after being washed with PBS 3 times. The intracellular ROS accumulation was measured by using a Becton-Dickinson fluorescence-activated cell analyzer while data analysis was performed with Modifit LT 2.0 (Becton-Dickinson, San Jose, CA, USA). About 1×10^4 cells were counted for each analysis. The fluorescence intensity was quantified with CELLQuest software (Becton-Dickinson, Mountain View, CA, USA).

Cell viability was assessed by MTT (3-(4,5-dimethylthiazolyl)-2,5-diphenyformazan bromide) reduction assay. PC12 cells were plated at density of 1×10^4 cells per 100 μL in 96-well plates. After incubation and treatment with OGFD and hydrogen peroxide, cells were treated with the 10 μL MTT solution (final concentration, 0.5 mg/mL) for 4 h. The dark blue formazan crystals formed in intact cells were solubilized with the lysis buffer (20% sodium dodecylsulfate in 50% aqueous *N*,*N*-dimethylformamide) and absorbance at 570/630 nm was measured with a microplate reader (Molecular Devices, Sunnylvale, CA, USA).

A flow cytometric method was used to assess the percentage of apoptotic nuclei [22]. The cells were harvested after treatment with OGFD and hydrogen peroxide for certain time, and then washed with PBS. The pellets were fixed in 70% (v/v) ethanol overnight at 4 °C. The cells were then resuspended in PBS (pH 7.4) containing 0.1% Triton X-100, 0.1 mM EDTA and 0.5 mg/mL RNase (Sigma, St. Louis, MO, USA) at 37 °C for 15 min and finally stained with 50 μg/mL propidium iodide (PI) for 1 h. Cell cycle analysis was performed by using a Becton-Dickinson fluorescence-activated cell analyzer and data analysis was performed with Modifit LT 2.0 (Becton-Dickinson, San Jose, CA, USA).

Cells treated with OGFD and hydrogen peroxide were stained with Hoechst 33342 (1 μg/mL) and propidium iodide (5 μg/mL) for 20 min at 37 °C [19]. Stained nuclei were observed, using fuorescence photomicroscope (Olympus, Japan).

Reduced glutathione (GSH) level was determined by the method described by Tietze (1969) [20]. Cells were collected and proteins were precipitated with 3.33% 5-sulfosalicylc acid dihydrate and removed by centrifugation. Measurements were carried out in microtiter plates by the addition of 25 μL of supernatant and 180 μL of assay mixture containing: NADPH (0.21 mM), 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) (0.6 mM), glutathione reductase (0.5 U/mL), ethylenediamine tetraacetic acid (EDTA) (6.3 mM) in phosphate buffer (0.125 M, pH 7.5). Absorbance was read at 405 nm with a plate reader (Victor, 1420 multilabel counter). Test sample values were calibrated against a standard curve of GSH.

PC12 cells treated with 800 μM hydrogen peroxide displayed intense fluorescence inside the cell after staining with DCF dye. The result is shown in the Fig. 2. When 50, 5, 0.5 μg/mL OGFD were added to the media respectively, intracellular ROS accumulation resulting from hydrogen peroxide treatment was reduced from 432.7 to 148.3, 161.3 and 229.4, respectively, which is determined by the changes in fluorescence intensity.

The protective role of OGFD which possess strong antioxidation activity was tested in the hydrogen peroxide-mediated cell death assay (Fig. 3). The viability of cells exposed to 800 μM hydrogen peroxide for 24 h without OGFD pretreatment was 33.8% of the control value. The viabilities of cells pretreated with OGFD at 5 μg/mL before exposed to hydrogen peroxide increased significantly to 80.3% of control value, while vita-