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In vitro protective effects of resveratrol against oxidative damage in human erythrocytes

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ABSTRACT

Resveratrol (RV) is a potent antioxidant, anticancer and anti-inflammatory agent. Its main target of action is the cell membrane; however, its effect on that of human erythrocytes has been scarcely investigated. With the aim to better understand the molecular mechanisms of the interaction of RV with cell membranes both human erythrocytes and molecular models of its membrane have been utilized. The latter consisted in bilayers of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE), representative of phospholipid classes located in the outer and inner monolayers of the erythrocyte membrane, respectively. Results by X-ray diffraction showed that RV produced a significant structural perturbation on DMPC bilayers, but no effects were observed in DMPE. Scanning electron (SEM) and defocusing microscopy (DM) observations showed that RV induced morphological alterations to the red cells from the normal discoid shape to echinocytes. These results imply that RV was located in the outer monolayer of the erythrocyte membrane. Results of its antioxidant properties showed that RV neutralized the oxidative capacity of HClO on DMPC and DMPE bilayers. On the other hand, SEM and DM observations as well as hemolysis assays demonstrated the protective effect of RV against the deleterious effects of HClO upon human erythrocytes.

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1. Introduction

Resveratrol (RV, trans-3,5,4′-trihydroxystilbene, Fig. 1) is a polyphenol non-flavonoid compound, particularly abundant in red grapes (*Vitis vinifera*) but it is also present in highly pigmented vegetables and fruits [1]. It has been proven to be a potent antioxidant [2], anticancer [3] and anti-inflammatory agent [4]. Functionally, RV belongs to phytoalexins, also called the plant antibiotics [5]. It exists as *cis*- and *trans*-isomers being the latter the preferred steric form, which is relatively stable if protected from high pH and light [6]. On the other hand, *cis*-resveratrol is not as biologically active as the *trans*- form, being the different molecular behavior of the two isomers dependent on their three-dimensional structure [7]. RV is considered to be one of the major constituents in red wine, which contains 6.5 mg/l resveratrol [8,9]. A primary impetus for research on RV was initiated from the observation that a low incidence of cardiovascular diseases may co-exist with a high-fat diet intake and moderate consumption of red wine, a phenomenon known as the French paradox [5,6,10,11]. Several diseases like cancer, inflammation, cardiovascular disorders, rheumatoid arthritis and neurodegeneration have been shown to be related to excessive generation of reactive oxygen species (ROS), which can cause damage to crucial biomolecules such as nucleic acids, proteins, polyunsaturated fatty acids, carbohydrates and cell membranes [2,12–14]. RV possesses three phenolic groups and acts as a free radical scavenger by transferring the proton from its phenolic group to the free radicals [7,12]. The molecular mechanism of action of polyphenol antioxidants would depend on their structure as well as of their capacity to intercalate into cell membranes where they would interact with lipid bilayers, acting as radical scavengers and thus protecting the membrane from oxidative stress [5, 12,15]. In spite of the health importance of RV and results showing that the main target of RV action is the cell membrane [1], its effect on cell membranes, particularly of the human erythrocytes, has been scarcely investigated [12]. Cell membrane is a diffusion barrier which protects the interior of the cell. Therefore, its structure and functions are susceptible to alterations as a consequence of interactions with chemical species.

With the aim to better understand the molecular mechanisms of the interaction of RV with cell membranes human erythrocytes and molecular models of its membrane were utilized. Human erythrocytes were chosen because of their only one membrane and no internal organelles which constitute an ideal cell system for studying interactions of chemical compounds with cell membranes [16]. On the other hand, although less

Abbreviations: RV, resveratrol; DMPC, dimyristoylphosphatidylcholine; DMPE, dimyristoylphosphatidylethanolamine; SEM, scanning electron microscopy; DM, defocusing microscopy

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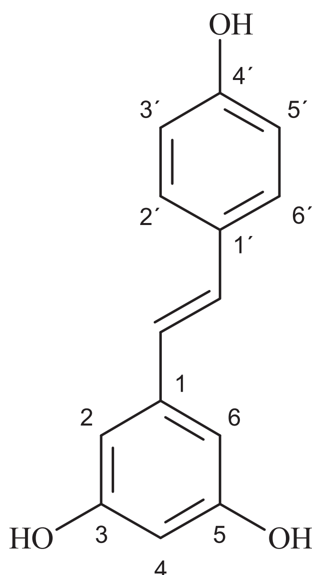

Fig. 1. Structural formula of resveratrol.

specialized than many other cell membranes they carry on enough functions in common with them such as active and passive transports, and the production of ionic and electric gradients to be considered representative of the plasma membrane in general. The molecular models of the erythrocyte membrane consisted in bilayers of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE), representative of phospholipid classes located in the outer and inner monolayers of cell membranes, particularly of the human erythrocyte, respectively [17,18]. The capacity of RV to perturb the bilayer structures of DMPC and DMPE was evaluated by X-ray diffraction; intact human erythrocytes were observed by scanning electron microscopy (SEM) and defocusing (DM) microscopy. These systems and techniques have been used in our laboratories to determine the interaction with and the membrane-perturbing effects of other chemical compounds, particularly of native plant extracts [19–22]. The antioxidant properties of RV were evaluated in the molecular models of the erythrocyte membrane and human erythrocytes in vitro exposed to the oxidative stress induced by hypochlorous acid. HClO is a powerful natural oxidant that damages bacteria, endothelial cells, tumor cells and erythrocytes [23–26].

2. Materials and methods

2.1. Chemicals

Synthetic DMPC (lot 140PC-246, MW 677.9) and DMPE (lot 140PE-60, MW 635.9) from Sigma (AL. USA) and *trans*resveratrol (lot SZBA154XV, MW 228.2) from Fluka (Sigma, AL, USA) were used without further purification. Composition of phosphate buffered saline (PBS) was 150 mM NaCl, 1.9 mM NaH_2PO_4, and 8.1 mM Na_2HPO_4, pH 7.4. Concentration of HClO from commercial samples was spectrophotometrically determined at 292 nm ($\varepsilon = 350\ M^{-1}\ cm^{-1}$) [27].

2.2. X-ray diffraction studies of DMPC and DMPE multilayers

The capacity of RV to perturb the structures of DMPC and DMPE multilayers was evaluated by X-ray diffraction. About 2 mg of each phospholipid was introduced into Eppendorf tubes which were then filled with 180 l of (a) distilled water (control), and (b) RV aqueous solutions in a range of concentrations (0.025–2.0 mM for DMPC and 1.0–10.0 mM for DMPE experiments). The specimens were shaken, incubated for 15 min at 30 °C and 60 °C with DMPC and DMPE, respectively and centrifuged for 20 min at 2500 rpm. Samples were then transferred to 1.5 mm diameter special glass capillaries (Glas-Technik&Konstruktion, Berlin, Germany) and X-ray diffracted utilizing Ni-filtered CuKα radiation from a Bruker Kristalloflex 760 (Karlsruhe, Germany) X-ray system. Specimen-to-film distances were 8 and 14 cm, standardized by sprinkling calcite powder on the capillary surface. The relative reflection intensities were obtained in an MBraun PSD-50 M linear position-sensitive detector system (Garching, Germany); no correction factors were applied. The experiments were performed at 18 ± 1 °C, which is below the main phase transition temperature of both DMPC and DMPE. Higher temperatures would have induced transitions onto fluid phases making the detection of structural changes harder. Each experiment was performed in triplicate.

2.3. Scanning electron microscopy (SEM) studies on human erythrocytes

Five blood drops (100 μl) from a human healthy donor not receiving any pharmacological treatment were obtained by puncture of the ear lobule and received in an Eppendorff tube containing 100 μl of heparin (5000 UI/ml) in 900 μl of phosphate buffer saline (PBS), pH 7.4. Red blood cells were then centrifuged (1000 rpm × 10 min),washed three times in PBS, resuspended in PBS containing RV in a range of concentrations and then incubated at 37 °C for 1 h, period in line with the larger effects induced by compounds on red cell shape [28,29]. Controls were cells resuspended in PBS without RV. Specimens were fixed overnight at 4 °C by adding one drop of each sample to plastic tubes containing 500 μl of 2.5% glutaraldehyde in distilled water, reaching a final fixation concentration of about 2.4%. Samples were washed twice in distilled water and centrifuged (1000 rpm × 10 min.); about 20 μl of each sample were placed on siliconized Al glass covered stubs, air-dried at room temperature, gold coated for 3 min at 13.3 Pa in a sputter device (Edwards S 150, Sussex, England, and examined in a scanning electron microscope (JEOL JSM-6380LV, Japan)). Data were expressed as mean ± standard deviation of 50 cell counts.

2.4. Optical and defocusing microscopy (DM) studies of human erythrocytes

Erythrocyte shapes were visualized and then analyzed through three dimensional reconstructions using defocusing microscopy (DM). DM is an optical technique based on the visualization and analysis of contrast images of transparent objects seen out of focus using a bright field microscope. Red blood cells (RBC) were obtained from a healthy donor under no pharmacological treatment. Cells were centrifuged (1000 rpm for 10 min) and washed three times with PBS pH 7.4 with 1 mg/ml of bovine serum albumin (BSA). RBC solution was prepared diluting the washed blood 20 times in a solution of PBS and BSA. RV solution was prepared in the same preparation of PBS and BSA. In order to carry out the analysis, 1.7 ml of RBC diluted solution was placed in an acrylic cuvette, and visualized at the optical microscope. After that, a morphologically normal erythrocyte was selected by using software [30] and the concentration of RV was increased. In order to make three-dimensional reconstructions, two images were captured in the defocus positions +1 and −1 μm at 20 μM of RV with or without HClO in the RBC solution. The contrast of the images was obtained and three-dimensional shape reconstruction was directly calculated [30,31].

2.5. Hemolysis assays

Red blood cells (RBC) were obtained from a healthy consenting donor. 10 ml of heparinized blood was centrifuged (EYDAM, Germany) at 2500 rpm for 10 min. After removal of plasma and buffy coat, the RBC were washed three times with PBS at room temperature, and resuspended in PBS three times its volume for subsequent analyses [32]. RBC (10% v/v) were incubated in a shaking bath for 15 min. at 37 °C in PBS in the presence of increasing concentrations of RV in a final 1.5 ml volume. After cooling, increasing concentrations of HClO in PBS were added and

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