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Original Articles

Identification of the factors responsible for the *in vitro* pro-oxidant and cytotoxic activities of the olive polyphenols oleuropein and hydroxytyrosol

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ABSTRACT

The olive polyphenols oleuropein and hydroxytyrosol were reported recently to produce extracellular hydrogen peroxide (H_2O_2) under standard culture conditions. The precise factors responsible for this production and the conditions promoting or retarding it are critical for the interpretation of the *in vitro* results. In this study, a systematic evaluation of the components of the most commonly used culture media revealed that sodium bicarbonate is the defining cause for the production of H_2O_2 by these polyphenols. The produced H_2O_2 caused extensive oxidative DNA damage and significant decrease in cell viability of cancer (MDA-MB-231) and normal (MCF-10A, STO) cells alike. Sodium pyruvate and the antioxidant N-acetyl cysteine (NAC) totally reversed these effects. Therefore, we conclusively identified the culture conditions that promote H_2O_2 production by these polyphenols, producing artifacts that may be misinterpreted as a specific anticancer activity. Our findings raise considerable questions regarding the use of culture media with sodium bicarbonate or sodium pyruvate as components, for the *in vitro* study of these and possibly other plant polyphenols.

1. Introduction

Oleuropein, the ester of hydroxytyrosol (3,4-dihydroxyphenylethanol) to elenolic acid, is the main polyphenol of olive fruit, oil and leaf, producing the bitter taste, [1] with its levels being decreased with the ripening of the fruit [2]. Oleuropein has been reported to possess strong antioxidant and free radical scavenger activity [3], antimicrobial [4], hypoglycemic [5,6], anti-toxoplasmosis [7], antiviral [8–10], antimycoplasmal [11], platelet anti-aggregant [12] and hypolipidimic [13] activities. Hydroxytyrosol, the metabolite of oleuropein, has also been reported to possess similar activities [14–18]. Oleuropein and hydroxytyrosol have also been reported to act as potent selective anticancer compounds in both cancer cell lines and mouse tumor models [19,20].

Hydroxytyrosol was identified recently to generate H_2O_2 in the Roswell Park Memorial Institute (RPMI) medium that was responsible for the induction of apoptosis in HL-60 cells [21]. Oleuropein, however, was shown to possess a peculiar behavior; although it produced significant amounts of H_2O_2, its pro-apoptotic effect on HL-60 cells was rather weak [22]. The instability of hydroxytyrosol and the generation of H_2O_2 was further demonstrated in other commonly used culture media like minimum essential medium (MEM) and Dulbecco's Modified Eagle Medium (DMEM) [23]. Other polyphenols of tea, apples and wine have also been found to be unstable in the culture media and produce significant levels of H_2O_2. More specifically, gallic acid, epigallocatechin gallate (EGCG), epigallocatechin and phenolic acids, were demonstrated to generate high levels of H_2O_2 in DMEM. Catechin and quercetin were observed to produce lower, but still significant, levels of H_2O_2 in the cell culture medium [24–26].

The ability of oleuropein and hydroxytyrosol to produce H_2O_2 has been previously reported by others [21–23]. However, the exact conditions and the mechanism of H_2O_2 production remain unknown. Specifically, it is not known whether the H_2O_2 production is strictly dependent on the chemical properties of the compound, the culture media components, other cell culture conditions, or a combination of these. More importantly, it is unknown if the cell-killing effects of the olive polyphenol-generated H_2O_2 are limited to cancer cells, or affect equally normal cells.

The aims of this study were to identify the precise conditions that lead to the H_2O_2 production by oleuropein and hydroxytyrosol, and to determine if cancer cells are more susceptible to the

Abbreviations: Amplex® Red reagent, 10-acetyl-3,7-dihydroxyphenoxazine; DMEM/F12, Dulbecco's modified Eagle medium-F12 mixture; EBSS, earle's balanced salt solution; FBS, fetal bovine serum; FOX, ferrous oxidation-xylenol orange; H_2O_2, hydrogen peroxide; MEM, minimal essential medium; NAC, N-acetyl cysteine; $NaHCO_3$, sodium bicarbonate; 8-oxo-dG, 7,8-dihydro-8-oxo-2′-deoxyguanosine.

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deleterious effects of the produced H_2O_2. We explored the capacity of oleuropein to produce H_2O_2 in the most commonly used culture media (i.e. DMEM, MEM and DMEM/F12). We have also evaluated the contribution of the MEM medium components to the production of H_2O_2, as well as the contribution of the culture conditions (i.e. pH). Our results will enable cancer researchers to control the levels of H_2O_2 produced during the *in vitro* testing of plant polyphenols, and to design more meaningful *in vivo* studies.

2. Materials and methods

2.1. Materials and cell cultures

Human breast cancer MDA-MB-231 cells, MCF-10A "normal" immortalized breast cells and mouse embryonic fibroblasts STO cells were obtained from American Type Culture Collection (Rockville, MD, USA). MDA-MB-231 and STO cells were cultured in DMEM (Invitrogen, Carlsbad, California, USA) containing 4500 mg/l glucose and supplemented with 10% (v/v) FBS and 1% antibiotic/antimycotic (100×) (Invitrogen, Carlsbad, California, USA), and incubated under standard conditions (37 °C, 5% CO_2), in a humidified atmosphere. MCF-10A cells were cultured in DMEM/F12 (Invitrogen, Carlsbad, California, USA) or DMEM medium supplemented with 100 ng/ml cholera toxin (Biomol, Hamburg, Germany), 0.5 μg/ml hydrocortisone (LKT Laboratories, Minnesota, USA), 10 μg/ml insulin (Roche Applied Science, Penzberg, Germany), 20 ng/ml EGF, 5% (v/v) horse serum and 1% antibiotic/antimycotic solution (100×) (Invitrogen, Carlsbad, California, USA).

2.2. Isolation oleuropein

Oleuropein was isolated from *Olea europaea* leaves as previously described [27]. Briefly, air dried and pulverized leaves (1 kg) were extracted by mechanical stirring for 12 h with acetone (2 × 2.5 l). The solvent was evaporated completely and washed with 1 l of a mixture of dicloromethane: methanol 98/2. The insoluble material (50 g) was separated, dried and subjected to medium pressure chromatography with normal silica gel 60 Merck (15–40 mm), using the dichloromethane methanol gradient as the eluent to extract pure oleuropein (2.5 g). The purity of oleuropein was 99% using analytical RP-HPLC with a Thermo Finnigan Spectra system (column: LiChrosob RP-18, 250 × 4.0 mm, 5 μm, elution solvent water acetonitrile gradient, flow: 1 ml/min, UV-detection: 254 nm). The purified oleuropein was entirely in the glucoside form and was free of any aglycone. Hydroxytyrosol was isolated from olive oil mill waste water, according to a previous described methodology [28]. Oleuropein and hydroxytyrosol were dissolved in dH_2O (Invitrogen, Carlsbad, California, USA), sterilized by filtration with 0.22 μm filters (Sartorius Stedim Biotech, GmbH, Goettingen, Germany) and kept at −20 °C in the dark. Dilution into culture medium was made just before use. The chemical structures of oleuropein and hydroxytyrosol are shown in Fig. 1.

2.3. Cell viability assay

Cells (5×10^3/well) were seeded overnight in 96 well microtiter plates. Oleuropein or hydroxytyrosol were added at concentrations ranging from 5 to 100 μg/ml for oleuropein and 3–100 μM for hydroxytyrosol in new DMEM medium and incubated at 37 °C, 5% CO_2 for 24 h. After treatment, the medium was removed and cells were washed with phosphate buffer solution (PBS) (Invitrogen, Carlsbad, California, USA), and fixed with 10% formalin (Sigma Aldrich, Steinheim, Germany). Crystal violet (Sigma Aldrich, Steinheim, Germany), was added for 15 min followed by washing with dH_2O. Plates were dried overnight. The stain was dissolved by 10% acetic acid (Fisher Scientific, Loughborough, UK) and measured spectrophotometrically at 570 nm using a Perkin–Elmer LS50 spectrofluorimeter. Sodium pyruvate (Invitrogen, Carlsbad, California, USA) and NAC (Sigma Aldrich, Steinheim, Germany) were added 1 h before treatment at final concentration of 1 mM. Experiments were performed in the continuous presence of these reagents.

2.4. Measurement of the H_2O_2 production

The production of H_2O_2 in MEM, DMEM, DMEM/F12 with and without supplements and MEM components (Invitrogen, Carlsbad, California, USA), was assessed by the ferrous oxidation-xylenol orange (FOX) assay and by the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen, Carlsbad, California, USA). For the FOX assay, oleuropein and hydroxytyrosol were incubated in phenol-red free culture media or components for different time points at 37 °C, 5% CO_2 or 37 °C, atmospheric CO_2. 20 μl of sample was added to 200 μl of FOX reagent containing 125 μM xylenol orange, 100 mM sorbitol, 250 μM ammonium Miron sulfate in 25 mM H_2SO_4 (Sigma Aldrich, Steinheim, Germany). For the Amplex Red kit, phenol-red free culture media or medium components were incubated with oleuropein at 37 °C, 5% CO_2. 50 μl of sample was added to 50 μl of Amplex red reagent with HPR enzyme diluted in PBS. For both of the methods, H_2O_2 was used as positive control and media or components without oleuropein or hydroxytyrosol, as negative controls. The H_2O_2 production was measured spectrophotometrically at 570 nm after 30 min incubation at room temperature. The concentration of H_2O_2 was derived from a standard curve obtained by adding different concentrations of H_2O_2 in dH_2O.

2.5. Detection of 8-oxo-DG by Immunocytochemistry

DNA oxidation was assessed by staining the cells with the monoclonal antibody Anti-8-oxo-dG (Clone 2E2) (Trevigen, Gaithersburg, USA) which detects the oxidized derivatives of deoxyguanosine. Cells were plated on cover slips in six well plates and the next day, were incubated with 100 μg/ml oleuropein in the presence or absence of 1 mM sodium pyruvate for 1 h. Untreated cells were used as negative control and H_2O_2 (50 μM) as positive control. The staining of the oxidized guanines (8-hydroxy-2′-deoxyguanosine) was performed according to the manufacturer's protocol. Fluorescence was visualized and photographed under the fluorescence microscope (Leica DM IL, Wetzlar, Germany). Cells with DNA oxidative damage had intense green color. DAPI staining was used as control.

2.6. Alkaline single-cell gel electrophoresis (comet) assay

DNA damage was assessed by the single cell gel electrophoresis assay under alkaline conditions, with the CometAssay kit from Trevigen (Gaithersburg, USA). Cells were treated with oleuropein (100 μg/ml) for 2 h. Untreated cells were used as negative control and H_2O_2 treated cells (100 μM, 20 min at 4 °C) as positive control. The cells were harvested by trypsinization, resuspended in PBS, mixed with low melting agarose and transferred on comet slides. Cell lysis, electrophoresis and staining were performed as described in the Trevigen instruction manual. Cells were stained with Sybr Green and examined under the fluorescence microscope (Leica DM IL, Wetzlar, Germany). Scoring was performed with the TriTek CometScore Freeware using the tail moment.

2.7. Data analysis

The graphs were designed with the GraphPad Prism 5 software (San Diego, CA, USA). The data are the Mean + SD of three independent experiments. Statistical analysis was performed by the Student's two-tailed *t*-test. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Oleuropein produces H_2O_2 in common culture media

When the olive polyphenol oleuropein was incubated in DMEM or MEM media at a concentration of 100 μg/ml, under standard cell culture conditions (37 °C, 5% CO_2), substantial amounts of H_2O_2 were produced as shown in Table 1. In contrast, only minor

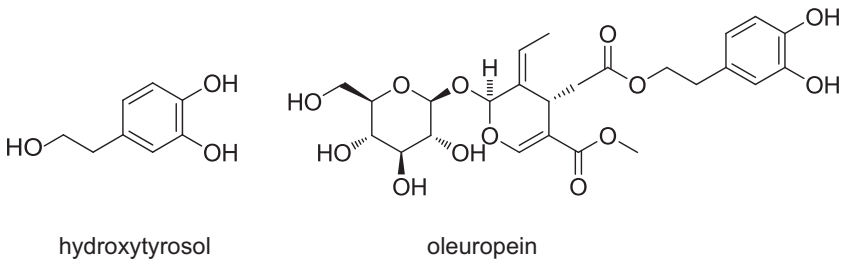

Fig. 1. Chemical structures of hydroxytyrosol and oleuropein.