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Study of interactions between phenolic compounds and H_2O_2 or Cu(II) ions in B14 Chinese hamster cells

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Abstract

The antioxidant and prooxidant effects of tannic, ellagic and gallic acids (1–60 μM) in the presence of hydrogen peroxide (40 and 100 μM) or copper ions (50 μM) on B14 Chinese hamster cells were examined.

The fluorescence probe DCFH-DA (dichlorofluorescein-diacetate) was used to analyse the levels of reactive oxygen species. This method showed the reduction in oxidation of DCF (dichlorofluorescein). It indicates that antioxidant capacity of tested polyhenols is decreased in the presence of hydrogen peroxide or copper ions.

Spectrophotometric assay with Ellman's reagent was used to determine SH-groups. The experimental results revealed the oxidative modification of proteins after exposure to polyphenols at concentrations above 15 μM. Additional incubation with H_2O_2 or Cu^{2+} ions showed the prooxidant activity of tested complexes also for polyphenols used at a concentration of 1 μM.

Fluorescence method with Hoechst 33258/propidium iodide dyes was used to study apoptotic and necrotic cell death. The obtained data demonstrated that polyphenols alone, as well as in the presence of hydrogen peroxide or copper ions, can induce DNA fragmentation.

Keywords: Phenolic acids; Thiol groups; Oxidative stress; Antioxidant activity; Apoptosis

1. Introduction

Tannic acids and its two derivatives, ellagic and gallic acids, represent a highly heterogeneous group of water-soluble polyphenolic compounds. They are produced in many plants as secondary metabolites in the cytoplasm and are considered to provide protection against herbivores (Fickel et al., 1999). They have also been reported to produce other physiological effects such as immune response, hepatotoxicity and lipid metabolism (Chung et al., 1998). Therefore, polyphenols have received attention due to the fact that they are biologically significant as antioxidants, anticarcinogens or antimutagens. Recent interest has concentrated on their likely health benefits as reducing agents that may play a role in the protection of body tissues against oxidative stress (Papadopoulou and Frazier, 2004).

Reactive oxygen radicals have a noxious effect on cells, and it is believed that free radical damage is involved in the etiology of several diseases. The radicals are a by-product of various endogenous processes that can be stimulated by external factors, such as irradiation and other agents such as xenobiotics and polyphenols. Antioxidants protect against these radicals, and it is important to balance an enhanced radical production with a sufficient supply of antioxidants (Arts et al., 2001).

The biological, pharmacological and medical properties of tested phenolic acids have been extensively reviewed. These polyphenols are reported, in addition to their free radical scavenging activity, to have multiple biological activities including vasodilatory, anticarcinogenic, antiinflammatory, antibacterial, immune-stimulating, antiallergic, antiviral and estrogenic effects (Rice-Evans et al., 1996). Their chemistry

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is predictive of free radical activity because the reduction potentials of polyphenols are lower than those of alkyl peroxyl radicals and the superoxide radical, which means that polyphenols may inactivate these species and prevent the deleterious consequences of their reactions (Jovanovic et al., 1992). Recently it has been shown that polyphenols, besides their scavenging activity, can act as prooxidants. Kondo et al. (1999) observed that these chemicals showed various cytotoxic effects on tissues and cells. This suggests that their biological action against free radicals may not be very simple.

In our previous studies (Labieniec and Gabryelak, 2003, 2005), we clarified that phenolic acids had toxic effects on proteins and DNA. They induced protein oxidation and caused DNA single and double-strand breaks. Therefore, in this paper, we present the continuation of our investigation with chosen polyphenols on B14 Chinese hamster cells. Although hydrogen peroxide by itself is a relatively innocuous molecule, it may generate far more reactive species such as hydroxyl radicals (Ciolino and Levine, 1997). Moreover, it is well known that a number of metals have carcinogenic potentials (Kawanishi et al., 2002). Therefore, we have complemented our experiments by interactions of tested phenols with H_2O_2 and Cu^{2+} ions. Thus, we could show that these acids, very promising natural compounds among antioxidant molecules, can also have a strong prooxidant effect. Therefore, based on our data, we suggest that tannic, ellagic and gallic acids are compounds whose activities require further investigation.

2. Material and methods

2.1. Chemicals

Tannic acid, gallic acid and 5,5′-dithiobis (2-nitrobenzoic acid) (Ellman's reagent) were obtained from Sigma–Aldrich (Germany). Ellagic acid, DCFH-DA (dichlorofluorescein-diacetate), Hoechst 33258 and propidium iodide were purchased from Sigma (St. Louis, MO, USA). All other reagents and solvents were of the highest analytical reagent grade.

2.2. Cell preparation

Chinese hamster fibroblasts (B14-cell line) were purchased from Child Health Centre in Warsaw (Poland). Monolayer cultures were grown in Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum, essential and non-essential amino acids, with 100 U/ml streptomycin and 2 mM L-glutamine. The cells were maintained at 37 °C in an atmosphere of 5% CO_2 and 95% air with more than 95% humidity.

2.3. Chemical treatment of cells

Tannic acid and gallic acid were taken from a stock solution (200 μM) in distilled water, ellagic acid was taken from a stock solution (200 μM) in 1% ethanol. The phenols were added to the suspension of cultured cells to give a final concentration in the range 1–60 μM. Hydrogen peroxide was taken from a 200 μM stock solution and $CuSO_4$ was taken from a 100 μM stock solution and added to the cell suspension at a final concentration of 40, 100 and 50 μM, respectively. The control cells were treated with PBS (pH 7.4). The cells were incubated for 1 h with the phenolic acids and in combination with H_2O_2 or $CuSO_4$ at 37 °C.

2.4. Cell viability

The viability of the cells was measured by Trypan blue exclusion assay. The B14 Chinese hamster cells were incubated for 1 h at 37 °C with polyphenols at concentrations in the range 1–60 μM, washed and suspended in PBS (pH 7.4). An equal volume of 0.4% Trypan blue reagent was added to the cell suspension and the percentage of viable cells was evaluated under a field microscope. The viability of the cells was calculated as the percent ratio of the relevant control. In general, the concentrations that caused cell viability to drop below 70% were considered to be too cytotoxic and were not assessed.

2.5. Analysis of antioxidant properties

DCFH-DA (2′-7′-dichlorofluorescein-diacetate) was dissolved in 5 mM DMSO and stored at 4 °C according to the procedure of Festa et al. (2001). Then, the fluorescence probe was hydrolyzed with 3 mM NaOH for 15 min and added to the cells (3×10^5 cells/ml) at a final concentration of 0.25 μM. After 1 h of incubation of the cells with the DCFH (2′-7′-dichlorofluorescein) and with polyphenols alone (1, 30 and 60 μM) and/or with H_2O_2 (40 and 100 μM), and/or with Cu^{2+} ions (50 μM) in PBS, the fluorescence intensity of DCF was detected fluorimetrically using a Perkin–Elmer LS-50 B spectrofluorimeter. The excitation and emission wavelengths were set at 480 and 530 nm, respectively.

2.6. Determination of SH-groups

To determine the total SH-group determination, cells (1×10^6) were exposed to tannic, ellagic and gallic acids at concentrations of 1, 15 and 60 μM and/or with H_2O_2 (40 and 100 μM), and/or with Cu^{2+} (50 μM) for 1 h. After incubation, cells were washed twice with PBS, collected by centrifugation at $3000 \times g$ for 10 min and resuspended in 1 ml 0.5 M phosphate buffer, pH 7.8, containing 0.1% SDS. Then, 25 μl Ellman's reagent (5 mM) was added and the amount of thiol groups was measured spectrophotometrically, using the excitation coefficient of 13.6 mM^{-1} cm^{-1} (412 nm) according to the procedure of Zavodnik et al. (2004).

2.7. Hoechst 33258/propidium iodide staining assay

The percentage of changed cells (apoptotic and necrotic cells) was evaluated using the fluorescence method with Hoechst 33258/propidium iodide dye described by Zhang et al. (1999). An aliquot of 100 μl cell suspension (1×10^6) previously exposed to phenolic acids at concentrations of 1, 5 and 60 μM and/or with H_2O_2 (40 and 100 μM), and/or with Cu^{2+} ions (50 μM) was mixed with 5 μl mixture of Hoechst 33258/propidium iodide (Ho 33258 – 0.13 mM; PI – 0.23 mM) and incubated for 5 min at 37 °C. After that, the mixture of 20 μl stained cells was put on a microscope slide and different types of cells (including apoptotic and necrotic cells) were counted using an Olympus microscope IX 70 (Japan). Three hundred images were randomly selected from each sample. Each data point of the initial DNA damage in the graphic presentation represents the mean ± S.D. of three individual experiments.

2.8. Statistical analysis

The values were expressed as means ± S.D. Data from three or six individual experiments were pooled and the statistical parameters were calculated by Normal distribution. Statistical differences between the means were assessed by ANOVA I. The statistical significance between the control and treated groups was also evaluated by Student's T-test. $P < 0.05$ was accepted as the statistically significant level.

3. Results

3.1. Cell viability

The cytotoxic effect of polyphenols (1–60 μM) was evaluated by a Trypan blue exclusion assay. The cell viability was