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Antioxidative and oxidative changes in the digestive gland cells of freshwater mussels *Unio tumidus* caused by selected phenolic compounds in the presence of H_2O_2 or Cu^{2+} ions

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Abstract

Research on biomarkers as early bioindicators of perturbation in populations and individuals has received increasing interest during recent decades. These ecotoxicity studies allow us to measure the impact of environmental stressors and to monitor and evaluate the degradation or restoration of systems. In the present study we used bivalve molluscs (mussels), which are sensitive biomarkers of aquatic ecosystem pollution, to assess the effects of three polyphenols: tannic acid, ellagic acid and gallic acid. These compounds were used in the 1–60 μM concentration range, alone and in the presence of H_2O_2 (40 and 100 μM) or Cu^{2+} ions (50 μM).

The fluorescence probe dichlorofluorescein-diacetate (DCFH-DA) was used to measure reactive oxygen species (ROS). The oxidation of DCFH-DA to the fluorescent DCF (dichlorofluorescein) by the phenolic compounds was investigated spectrofluorimetrically. The results showed that the polyphenols tested can act as antioxidants against the ROS present in the digestive gland cells, but their activity is decreased after incubation with hydrogen peroxide or copper ions.

SH-groups were determined spectrophotometrically using Ellman's reagent. The results showed that oxidative modification of proteins increased in a concentration-dependent manner in cells incubated with polyphenols (above 15 μM) alone. Incubation of the cells with phenolic acids and H_2O_2 or Cu^{2+} ions revealed that the phenolic acids had prooxidant properties in all concentrations used except for 1 μM tannic and ellagic acid and 40 μM H_2O_2.

DNA fragmentation was estimated by a fluorescence method using Hoechst 33258/propidium iodine binding. The data showed that the phenolic acids alone and in the presence of hydrogen peroxide or copper ions can induce apoptosis and necrosis.

The methods used and results obtained indicate that the polyphenols selected can act not only as antioxidants but also as prooxidants in digestive gland cells of *Unio tumidus*.

Keywords: Polyphenols; Oxidative stress; Antioxidant; Thiol groups; Apoptosis; Necrosis

1. Introduction

Biomarkers are biological parameters that provide measurements of behaviour, physiology, biochemistry, cell integrity, genomic structure and gene expression. They indicate either normal status or changes in individuals among the population studied. The use of biomarkers for environmental safety implies a thorough knowledge of their biological function (Vasseur and Cossu-Leguille, 2003). Freshwater mussels such as *Unio tumidus* are well suited for biomonitoring because they are reasonably sized, largely sessile, long-lived, widely distributed, available in large quantities and relatively tolerant to xenobiotics. They are often used to monitor water quality (Englund and Heino, 1995). Because mussels are known to accumulate various pollutants, they are used to assess early effects of pollution in water ecosystems, so they are interesting biological monitors (Vidal et al., 2002).

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The polyphenols examined in this study influence ecosystem function by affecting decomposition rates, nutrient cycling and herbivory. Their structural characteristics play an important role in regulating reactivity in ecologically important processes (Kraus et al., 2003).

Tannic acid and its two derivatives, ellagic acid and gallic acid, constitute a group of phenolic compounds that have received considerable attention with respect to their possible nutritional and physiological actions. There is growing evidence that these compounds may provide protection against microbial protein synthesis and protect ruminants from bloat. However, high levels produce adverse effects (decreased nutrient utilization, decreased animal productivity and death) in certain animals (Becker and Makkar, 1999). It is very well known that several phenolic substances in plants are cytotoxic and genotoxic and their consumption correlates with tumour incidence (Yen et al., 2001). Therefore, understanding the health benefits and/or potential toxicities of these plants is important.

Polyphenolic compounds are widely distributed in plants and are considered to be dietary antioxidants. Their ability to protect cells from oxidative stress has been well documented (Sergediene et al., 1999; Dauer et al., 2003). They are active against directly and indirectly acting mutagens in various *in vitro* and *in vivo* systems. Moreover, phenolic acids show a wide and contradictory spectrum of activities: antitumour, antiviral, antibacterial and cardioprotective (Szaefer et al., 2003). Most of these compounds have similar properties to humic substances, especially the ability to chelate many metal ions (Cruz et al., 2000). However, there have been recent reports (Jacobi et al., 1998; Hotta et al., 2001) that polyphenols have cytotoxic effects on some types of cells. These authors suggest that the relationship between the antioxidant activity and oxidation potential of phenolic acids is not necessarily simple and needs further clarification. In our previous papers (Labieniec et al., 2003; Labieniec and Gabryelak, 2004, 2006) we also demonstrated that selected polyphenols have cytotoxic and genotoxic effects on the digestive gland cells of *U. tumidus*. In this report, an extension of our previous studies, we describe the effects of these compounds alone or in the presence of H_2O_2 or Cu^{2+} ions. Vlyssides and Israilides (1997) observed that organic compounds in water are difficult to break down and are considered highly toxic pollutants; on the basis of this work, we also considered the influence of selected polyphenols on mussel proteins and DNA. One possible mechanism by which they effect cytotoxic damage is through autooxidation via a semiquinone radical during the formation of reactive oxygen species. The toxicity of these radicals is elevated in the presence of Cu^{2+}. During these reactions Cu^{2+} is reduced to Cu^{1+} and subsequently reoxidized in a Fenton-like reaction with H_2O_2 as well as with O_2 leading to the production of $O_2^{\cdot-}$, $^{\cdot}OH$ and other reactive oxygen species (Li and Trush, 1994). Therefore, we investigated whether the polyphenols used can be oxidized by Cu^{2+} or H_2O_2, inducing oxidative stress in the mussel cells examined. At the same time, we checked their antioxidant activities and tried to explain the mechanism of their double action.

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 iodine were purchased from Sigma (St. Louis, MO, USA). All other reagents and solvents were of the highest analytical reagent grade.

2.2. Cell preparation

Adult mussels (*U. tumidus*) were collected from the Pilica river, Poland between October and December 2005. They were acclimatised to laboratory conditions in a 15 l aquarium in air-saturated water (O_2 12.2 mg/l, pH 8.25, temperature 5–6 °C) for at least 7 days before the experiment. Cells were isolated according to the procedure of Birmelin et al. (1999). Briefly, pieces of digestive gland tissue were removed and carefully washed twice with cold phosphate-buffered saline (PBS, pH 7.4), then transferred to a flask for treatment with 0.1% trypsin at 14 °C. The tissue was dissociated for 2 h, then the cells were washed twice in PBS (pH 7.4). The concentration of the single-cell suspension was adjusted with PBS to 1×10^6 cells/ml for determination of SH-groups, apoptosis and necrosis, or to 3×10^5 cells/ml for determination of antioxidant properties.

2.3. Cell viability

Cell viability was measured by trypan blue exclusion. Digestive gland cells were incubated for 1 h at 5 °C at polyphenol concentrations in the range 1–60 μM, washed and suspended in PBS (pH 7.4). An equal volume of 0.4% trypan blue reagent (TBD) was added and the percentage of viable cells was evaluated under a field microscope. Cell viability was calculated as the percentage of referent control. In general, polyphenol concentrations that resulted in $\leqslant$70% relative viability were considered too cytotoxic and were not evaluated.

2.4. 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). The fluorescence probe was hydrolyzed with 3 mM NaOH for 15 min and added to the cells (3×10^5 cells/ml) to a final concentration of 0.25 μM. After 1 h incubation with polyphenols (1, 30 and 60 μM) alone, and/or with H_2O_2 (40 and 100 μM), and/or with Cu^{2+} (50 μM) in PBS, the fluorescence