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THE EFFECT OF PLANT PHENOLICS ON THE PROCESSES INVOLVED IN THE INITIATION AND PROMOTION OF CARCINOGENESIS: LIPID PEROXIDATION

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Phenolics, common plant constituents form up an important part of human diet and are considered potential chemopreventive agents. In the present study, three structurally diverse phenolic acids: protocatechuic, chlorogenic and tannic acid, were investigated for their ability to inhibit lipid peroxidation and induce antioxidant enzymes in mouse epidermis and in rat liver and kidney. All three phenolics significantly decreased the level of lipid peroxidation in the epidermal, hepatic and renal microsomes *in vitro*. The non-enzymatic lipid peroxidation was more affected. The most potent inhibitor of lipid peroxidation in all systems was tannic acid (IC_{50} = 2.5-13.0 μM). In addition, all tested compounds significantly enhanced the activities of cytosolic glutathione peroxidase (GPx), especially GPx II. Again, the most effective inducer of this enzyme was tannic acid which increased the level of enzyme activity by 100-300%. Our results indicate that the phenolic acids, ubiquitous food components, particularly those with higher degree of ring hydroxylation, may inhibit or modulate the oxidative damage in the cell.

INTRODUCTION

Lipid peroxidation is a highly destructive process which induces alterations in the structural integrity of biological membranes. The genotoxic products of lipid peroxidation may be important mediators of DNA damage associated with oxidative stress which leads to induction of carcinogenesis [Esterbauer, 1982]. Malondialdehyde (MDA) is the most abundant carbonyl compound and the major mutagenic and carcinogenic product generated by lipid peroxidation. The formation of MDA-DNA adduct in human liver was shown and its level was comparable to that of adducts formed by exogenous carcinogens [Chaudhary *et al.*, 1994]. These findings may suggest that MDA is involved in the initiation stage of carcinogenesis. On the other hand, peroxidation of phospholipids may affect also the tumor promotion, since the inflammation which is associated with the exposure to some tumor promoters, like phorbol esters in mouse skin, is a possible source of free radicals [DiGiovanni, 1992]. Thus, the control of lipid peroxidation and the modulation of cellular antioxidant enzymes is considered an important mechanism of chemical carcinogenesis inhibition.

Consistent with the involvement of oxidative stress in cancer induction and its subsequent development, efforts are being made to identify naturally occurring compounds which could prevent, slow and/or reverse cancer induction and its subsequent development [Zhao *et al.*, 2000]. Plant phenolics, found in many edible vegetables, nuts and fruits, make up an important part of human diet [Stoner & Mukhtar, 1995] and are considered promising cancer chemopreventive agents. Structurally diverse phenolics, protocatechuic acid, chlorogenic acid and tannic acid were reported to inhibit mutagenesis and/or tumorigenesis induced by polycyclic aromatic hydrocarbons (PAHs) and heterocyclic amines [Ohnishi *et al.*, 1997; Nakamura *et al.*, 2000; Mukhtar *et al.*, 1988]. Several mechanisms of their anticarcinogenic activity were proposed, including electrophile radical trapping and modulation of arachidonic metabolism [Newmark, 1987]. Our earlier studies showed that phenolic acids can modulate the activities of cytochrome P450-mediated enzymes and inhibit the formation of PAHs-DNA adducts *in vivo* and *in vitro* [Baer-Dubowska *et al.*, 1997, 1998; Krajka-Kuźniak & Baer-Dubowska, 2003; Szaefer *et al.*, 2003; Ignatowicz *et al.*, 2003]. These phenolic acids were shown to scavenge free radicals formed *in vitro* by 2,2'-azoinobis (3-ethylbenzothiazoline 6-sulfonic acid) or 1,1-diphenyl-2-picrylhydrazyl [Riedl *et al.*, 2001; Kweon *et al.*, 2001; Toda *et al.*, 1985].

This research aimed at assessing the ability of three structurally diverse phenolic acids to inhibit lipid peroxidation in biological membranes and induction of antioxidant enzymes in mouse epidermis and in rat liver and kidney.

MATERIALS AND METHODS

Chemicals. Protocatechuic acid (97%), chlorogenic acid (95%), NADPH, ADP, cumene hydroperoxide, hydrogen peroxide, glutathione (GSH), glutathione reductase, bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Tannic acid (97%) was purchased from Aldrich-Chemie (Steinheim, Germany). All other compounds were readily available commercial products.

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ANIMALS AND TREATMENT

The animals, rats and mice, were housed in polycarbonate cages containing hardwood chip bedding. A standard pellet diet (Altromin GmbH, Germany) and distilled water were available without restriction.

Female Swiss mice seven to nine weeks old (25 g) were shaved on the dorsal side using surgical clippers two days before the treatment. Phenolic acids were applied topically to the shaved dorsal skin at the doses of 8 μmol and 16 μmol in 0.2 mL of acetone. A control group of mice was treated with acetone alone. Five mice were used for each experimental group. Twenty four hours after the last treatment, the animals were killed by cervical dislocation. After the skins were washed with cold water, they were removed and stored on ice. The epidermis was collected by scraping skins on a cold glass plate as described previously [Baer-Dubowska *et al.*, 1997].

Male Wistar rats (200 g) were given an intraperitoneal (i.p.) injection of 50 mg/kg protocatechuic acid (dissolved in olive oil) or 20 mg/kg and 50 mg/kg tannic acid (dissolved in buffered salt solution) twice a week. Control rats received only a vehicle (0.5 mL of olive oil or buffered salt solution, respectively). Rats were killed by decapitation twenty four hours after the last treatment. Livers and kidneys were immediately removed and homogenized. Microsomal and cytosolic fractions were prepared by differential centrifugation. Protein concentrations were determined by the method of Lowry *et al.* [1951] using bovine serum albumin as the standard.

All experiments were conducted according to the Regional Ethics Committee guidelines for animal experimentation.

Lipid peroxidation assay [Ohkawa *et al.*, 1979; Vaca *et al.*, 1988]. The incubation mixtures (1.5 mL, total volume) consisted of 0.15 mol/L Tris-HCl, 1 mmol/L KH_2PO_4 buffer (pH 7.4), microsomes (1-3 mg protein) and additionally 0.01 mmol/L $FeSO_4$ and 0.05 mmol/L L(+)-ascorbic acid (non enzymatic lipid peroxidation) or 4 mM NADPH, 4 mmol/L ADP, and 0.05 mmol/L $FeCl_3$ (enzymatic lipid peroxidation). Incubations were carried out aerobically at 37°C for 20 min (non-enzymatic lipid peroxidation) or for 60 min (enzymatic lipid peroxidation) with various concentrations of the tested compounds. Control incubations did not contain the phenolics tested. The level of MDA-like substances used as an index of lipid peroxidation was assayed with the 2-thiobarbituric acid using molar absorption coefficient of 1.56×10^5 $M^{-1}cm^{-1}$ with absorbance correction as described by Macfarlane [1981]. Results were expressed as nmol malondialdehyde equiv./mg protein. Per cent inhibition of lipid peroxidation at the concentration range of 1×10^{-3}-1×10^{-9} M phenolics were calculated and IC_{50} were estimated by linear regression.

Glutathione peroxidase assay. Glutathione peroxidase (GPx) activity was measured according to the procedure described by Mohandas *et al.* [1984]. The assay mixture consisted of 0.05 mol/L phosphate buffer (pH 7.0), 1 mmol/L EDTA, 1 mmol/L sodium azide, 1 unit of glutathione reductase, 1 mmol/L glutathione (GSH), 0.2 mmol/L NADPH, cytosol (0.4-0.7 mg protein of epidermis or 0.2-0.3 mg protein of liver and kidney), and either 0.25 mmol/L hydrogen peroxide or 1.5 mmol/L cumene hydroperoxide in the final volume of 1.0 mL. The disappearance of NADPH at 340 nm was recorded at 25°C. The activity with hydrogen peroxide as a substrate represented glutathione peroxidase I, and the difference between cumene hydroperoxide- and hydrogen peroxide-initiated activities represented glutathione peroxidase II. The activity of the enzyme was expressed as nmol of NADPH oxidized/min/mg protein.

Statistical analysis. Experimental groups were compared with their respective controls using ANOVA followed by Student's t-test. Differences were considered significant at $p \leq 0.05$.

RESULTS

Effect of phenolic acids on lipid peroxidation *in vitro*

The effects of protocatechuic acid, chlorogenic acid and tannic acid on enzymatic and non-enzymatic lipid peroxidation in rat liver microsomes and mouse epidermis are presented in Table 1. All tested compounds significantly decreased the level of lipid peroxidation in both tissues. The non enzymatic lipid peroxidation was more affected. The highest levels of inhibition were observed in the case of non-enzymatic lipid peroxidation in rat liver microsomes. The most potent inhibitor of lipid peroxidation in all systems was tannic acid (IC_{50} = 2.5-13.0 μmol/L), while protocatechuic acid was the least effective inhibitor (IC_{50} = 250-2 000 μmol/L).

TABLE 1. Inhibition of lipid peroxidation in rat liver microsomes and mouse epidermis IC_{50} (μmol/L).

Compound	Hepatic enzymatic lipid peroxidation	Hepatic non enzymatic lipid peroxidation	Epidermal non enzymatic lipid peroxidation
Protocatechuic acid	2 000	200	250
Chlorogenic acid	500	50	80
Tannic acid	13	3	2.5

Effect of phenolics on glutathione peroxidase I and II activities in mouse epidermis

As shown in Table 2, all tested compounds significantly enhanced the activities of cytosolic GPx. The most effective inducer of GPx I was tannic acid. The treatment with 8 μmol and 16 μmol of this compound resulted in a 3-fold increase in epidermal GPx I activity in comparison with acetone-treated control. Chlorogenic acid was the least effective inducer of this enzyme. The activity of GPx II was elevated mostly by protocatechuic acid. Topical application of this compound resulted in 156 and 422% increase of GPx II activity in a dose of 8 and 16 μmol, respectively. Tannic acid increased the activity of GPx II to a similar extent in both tested doses. Chlorogenic acid enhanced GPx II activity by 164 and 250% when applied in a dose of 8 and 16 μmol, respectively. The observed differences in comparison with the control group were statistically significant ($p \leq 0.05$).

TABLE 2. The effect of plant phenolics on glutathione peroxidase activity in mouse epidermis.

Compound	Dose [μmoles]	GPx I [nmol/min/ /mg protein]	GPx II [nmol/min/ /mg protein]
Acetone	0.2 mL	82.79 ± 2.27	58.66 ± 2.78
Protocatechuic acid	8	171.19 ± 12.78*	150.43 ± 7.48*
	16	195.32 ± 20.79*	306.43 ± 11.14*
Chlorogenic acid	8	154.37 ± 7.92*	155.02 ± 6.55*
	16	126.17 ± 6.17*	205.59 ± 21.45*
Tannic acid	8	230.15 ± 11.94*	209.63 ± 9.26*
	16	243.68 ± 18.06*	221.33 ± 9.89*

[a]Values are means ± SEM of 3 separate experiments run in triplicate; *Statistically different from control, p≤0.05

Effect of phenolics on glutathione peroxidase I and II activities in rat liver and kidney

The effects of i.p. administered protocatechuic acid and tannic acid on GPx I and II activities in rat liver and kidney were also investigated. Similarly to the results observed in mouse epidermis, both tested compounds enhanced the activity of GPx II to a higher extent (Table 3). Tannic acid was a more effective inducer of this enzyme than protocatechuic acid. The treatment of rats with tannic acid resulted in a similar pattern of induction of GPx II in liver and kidney (about 2.5-fold and 3.5-fold in a dose of 20 and 50 mg/kg b.w., respectively). The observed differences were statistically significant (p≤0.05), as compared to controls.

DICUSSION

A comparative analysis of the antioxidant effect on lipid peroxidation of the three naturally occurring phenolic acids in rat and mouse microsomes revealed that according to IC_{50} values their effectiveness could be classified as follows: tannic acid>chlorogenic acid>protocatechuic acid. The non-enzymatic, Fe^{+2}/ascorbate driven lipid peroxidation, was more affected than NADPH/ADP/Fe^{+3} driven reaction in all tissues studied. Tannic acid inhibited the non enzymatic lipid peroxidation to the extent comparable to classic synthetic antioxidant, BHT [Afanas'ev *et al.*, 1989]. In the initiation of NADPH-dependent lipid peroxidation, the cytochrome P450 reductase is involved, while the propagation step depends on reactive oxygen species (ROS). Lipid peroxidation driven by Fe^{+2}/ascorbate is ROS-dependent both at the initiation and propagation stage. Thus phenolic acids tested in this study might rather affect the ROS formation than inhibit cytochrome P450 reductase. This possibility is supported by the fact that the similar effect was observed also for ellagic acid in mice liver microsomes [Majid *et al.*, 1991]. The antioxidant activity of phenolic compounds is determined by their molecular structure and more specifically, by the position and degree of hydroxylation of their rings [Gordon *et al.*, 1990]. It was also suggested that the addition of methoxy group to *ortho* position increases the antioxidant activity by stabilization of phenoxy radical [Cuvelier *et al.*, 1992], while the addition of the hydroxyl group in the same position reduces the antioxidant activity. This could explain the low antoxidant activity of protocatechuic acid observed at *in vitro* test with β-carotene [von Gadov *et al.*, 1997]. The results of our present studies, although confirm the low antioxidant activity of protocatechuic acid in biological membranes, do not support this theory. None of the tested phenolic acids has methoxy group and their antioxidant activity correlated with the number of hydroxy groups. A similar relationship was found by Pratt [1980] for the natural antioxidants present in soybean and other oil seeds. Antioxidants may protect against the initiation as well a promotion stage of chemically-induced tumors. In mouse epidermis promotion is exerted by the application of phorbol esters like 12-*O*--tetradecanoylphorbol-13-acetate (TPA), which results also in ROS formation and a decrease in the activity of the ROS detoxifying enzymes. It was shown that selenium is an effective inhibitor of skin tumor promoting activity of TPA [Perchellet *et al.*, 1987]. Selenium is an important cofactor of GPx, the enzyme involved in detoxifying hydrogen peroxide and indirectly superoxide in the cell [Fridovich *et al.*, 1976]. Thus, the induction of GPx by protocatechuic acid and tannic acid, observed in the present study particularly in mouse epidermis, might be important for the inactivation of ROS generated by TPA and inhibition of tumor promotion. Antioxidants, however, have to be considered a double-edged sword in cancer control. It was reported that transgenic mice with overexpression of GPx or both GPx and superoxide dismutase showed an enhanced tumorigenic response to the application of 7,12--dimethylbenz[a]anthracene (initiator)/TPA (promoter) [Lu *et al.*, 1997]. This observation indicates that altered

TABLE 3. The effect of protocatechuic and tannic acids on glutathione peroxidase activity in rat liver and kidney.

Compound	Tissue	Dose [mg/kg b.w.]	GPx I [nmol/min/mg protein]	GPx II [nmol/min/mg protein]
Protocatechuic acid	Liver	0.5 mL of vehicle	119.08 ± 4.67	1384.38 ± 38.29
		50	370.88 ± 10.01*	2636.38 ± 91.20*
	Kidney	0.5 mL of vehicle	149.28 ± 11.01	226.14 ± 30.67
		50	223.55 ± 13.15	541.21 ± 12.71*
Tannic acid	Liver	0.5 mL of vehicle	418.33 ± 19.75	1213.74 ± 16.55
		20	670.09 ± 22.13*	2970.49 ± 177.26*
		50	805.39 ± 43.99*	4304.39 ± 134.90*
	Kidney	0.5 mL of vehicle	412.60 ± 57.46	569.10 ± 77.06
		20	832.80 ± 56.52*	1484.50 ± 81.25*
		50	1170.57 ± 125.62*	2051.98 ± 83.93*

[a]Values are means ± SEM of 3 separate experiments run in triplicate. *Statistically different from control, p≤0.05

ROS-detoxification enzyme levels might influence the process of carcinogenesis by modulating cell growth phenotype, and increasing resistance of cells with oxidative damage to repair processes.

CONCLUSIONS

Naturally occurring phenolic acids inhibit lipid peroxidation in biological membranes in tissue dependent manner. The extent of inhibition depends mainly on the number of hydroxy groups in the compound ring. Moreover, the most efficient inhibitor of lipid peroxidation like tannic acid are able to induce glutathione peroxidase *in vivo*.

The results of this study show that all phenolics significantly decreased the level of lipid peroxidation in all tested tissues *in vitro*. Tannic acid was the most efficient inhibitor. In addition, phenolic acids induced the activity of cytosolic glutathione peroxidase.

Our results indicate that the phenolic acids, ubiquitous food components, particularly those with higher degree of ring hydroxylation, may inhibit or modulate the oxidative damage in the cell. More detailed studies which are under way are necessary to establish the dose and way of administration in order to achieve anticarcinogenic effect.

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