Toxic Dose of a Simple Phenolic Antioxidant, Protocatechuic Acid, Attenuates the Glutathione Level in ICR Mouse Liver and Kidney

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It has previously been reported that a toxic dose of protocatechuic acid (PA), a naturally occurring simple phenolic antioxidant in dietary plant foodstuff, has a potential to enhance tumorigenesis and induce contact hypersensitivity in mouse skin. In this study, the modifying effect of a toxic dose of PA on the glutathione (GSH) level in mouse liver and kidney was examined. Intraperitoneal administration of PA (500 mg/kg) caused significant hepatic and nephrotic GSH depletion. Interestingly, slight but significant hepatotoxicity and nephrotoxicity, characterized by the enhancement of plasmic alanine aminotrasferase (ALT) activity and urea level, respectively, were also observed. The subchronic administration of PA (0.1% in drinking water) for 60 days showed not only a significant decrease in the GSH level in kidney but also a significant enhancement of ALT activity in plasma. The protective role of GSH for acute hepatotoxicity using GSH-depleted mice administered a GSH synthesis inhibitor buthionine sulfoximine was also demonstrated. Thus, it is suggested that overdoses of PA can disturb the detoxification of other electrophilic toxicants including ultimate carcinogens.

Keywords: *Protocatechuic acid; antioxidant; toxicity; mouse; glutathione*

Food phytochemicals showing antioxidant activities have mainly been recognized as most promising candidates for chemopreventers against oxidative stress-related diseases, including cancer, because they have been found to strongly inhibit oxidative reactions in vitro and in vivo. These compounds indeed have antimutagenic and anti-tumor-promoting activities and possess several other biological and physiological properties possibly related to anticarcinogenesis. On the other hand, some antioxidants exert not only weak antitumor-promoting activity but also carcinogenic activity in rodents when given at a high dose (*1*). For example, both artificial antioxidants, including 3-*tert*-butyl-4-hydroxyanisole, and naturally occurring compounds including caffeic acid have shown not only tumor-promoting activity in rat forestomach carcinogenesis but also induction of forestomach squamous cell carcinoma of rats (*1*). Although dramatic pharmacological and biological activities of naturally occurring antioxidants have so far been much focused on, documentation of their safety and toxicology has been limited except for initial studies on antioxidative vitamins (*2*).

Protocatechuic acid (PA; 3,4-dihydroxybenzoic acid) is one of the major benzoic acid derivatives from edible plants and fruits; it shows a strong antioxidative effect, 10-fold higher than that of α-tocopherol (*3*). PA at 100 ppm in repeated oral administration shows potent chemopreventive effects on colon and oral carcinogenesis in rats (*4*, *5*). The study on absorption and metabolism of cyanidin glucoside (CyG) demonstrated that PA, possibly derived from degradation of CyG (*6*), is actually present in the plasma of the CyG-fed rat and might contribute to the in vivo antioxidative activity of CyG (*7*). We have recently demonstrated the significant enhancement of mouse skin tumor promotion, inflammation, and oxidative stress by topical pretreatment with a high dose of PA (>1 μmol), whereas a relatively lower dose (16 nmol) attenuated these responses (*8*). The application of PA alone at a large amount also showed immunoinflammatory responses including contact hypersensitivity (*9*). The possibility that metabolism by dermal tyrosinase activity of PA to compound(s) lacking antioxidative properties and/or rather possessing the potential to enhance tumor development has also been suggested.

Glutathione (GSH), the major cellular antioxidant, is well-known to have diverse biological functions including protection of cells from damage by substances such as reactive oxygen species, free radicals, and reactive electrophiles including α,β-unsaturated carbonyl compounds. Our recent studies demonstrated that treatment with a high dose of PA alone for 3 h enhanced oxidative stress by reducing GSH levels in mouse skin, which was counteracted by a tyrosinase inhibitor, arbutin (*8*, *9*). These results strongly suggested that the tyrosinase-derived reactive quinone intermediates of PA, which reacts with nucleophilic residues of proteins including sulfhydryl groups or GSH, were involved in dermatotoxicity (Figure 1). GSH is, therefore, regarded as a protective regulator of PA-induced oxidative damage.

In the present study, we examined the modifying effect of toxic doses of PA on GSH levels in ICR mouse liver and kidney at acute and subchronic phases. We

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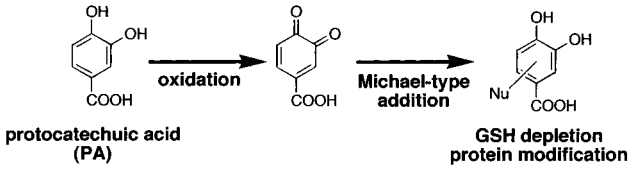

Figure 1. Toxic biotransformation of protocatechuic acid (PA).

also showed using GSH-depleted mice that GSH plays the negatively regulating role for acute hepatotoxicity.

MATERIALS AND METHODS

Chemicals. PA was purchased from Nacalai Tesque, Inc., Kyoto, Japan. Buthionine sulfoximine (BSO) was obtained from Aldrich Chemical, Co., Ltd. All other chemicals were purchased from Wako Pure Chemical Industries, Osaka, Japan.

Treatment of Animals. Female ICR mice (7 weeks old) were obtained from Japan SLC, Shizuoka, Japan. Mice used in each experiment were supplied with fresh tap water ad libitum and rodent pellets (MF, Oriental Yeast Co., Kyoto, Japan) freshly changed twice a week. Animals were treated in accordance with the Guidelines for Animal Experimentation of Kyoto University. One group was composed of five female ICR mice. In the acute toxicity experiments, the mice were dosed with PA or acetaminophen (AAP) at 50 or 500 mg/kg, intraperitoneal (ip), in 33% polyethylene glycol in saline at 37 °C. BSO was given as an ip dose of 800 mg/kg in 0.9% NaCl 2 h before PA administration. The mice were sacrificed 6 h after AAP or PA application. In the subchronic toxicity experiment, the mice were given drinking water containing PA or AAP (0.01 or 0.1%) for 60 days. Immediately after urine and blood had been collected according to methods reported previously (*10*), the mice were sacrificed and the livers and kidneys were removed. Blood was allowed to coagulate at room temperature, and the samples were centrifuged to obtain the serum. The livers and kidneys were weighed and homogenized in ice-cold PBS(−) (pH 7.4).

Determination of Toxic Parameters. The GSH content in each tissue was measured spectrophotometrically using a commercial kit (BIOXYTECH GSH-400 assay; OXIS International, Inc., Portland, OR) as reported previously (*8*, *11*). The activities of alanine aminotransaminase (ALT) and aspartate aminotransaminase (AST) were measured by a GPT-test kit and a GOT-test kit (Wako Pure Chemical Industries), respectively. The plasmatic urea level was quantified by a uric nitrogen B-test kit (Wako Pure Chemical Industries). These experiments were performed using the protocol outlined by the manufacturers. The urinary protein level was determined with the BCA protein assay reagent (Pierce) with bovine serum albumin as the standard.

Statistical Analysis. The data were analyzed with an analysis of variance (ANOVA) when necessary, followed by Fisher's test. Specific differences among treatments were examined using Student's *t* test (two sided), which assumed unequal variance.

RESULTS AND DISCUSSION

AAP, a well-known analgesic and antipyretic drug, is regarded as an appropriate positive control for acute toxicity in liver and kidney because a great number of studies of AAP toxicity have been documented. As shown in Figure 2, the ip administration of AAP and PA (500 mg/kg) led to a significant and dose-dependent decline of the hepatic GSH level by ~30% 6 h after administration. In contrast, the treatment with PA dramatically decreased the GSH level in kidney, whereas AAP had little effect. The ALT and AST activities, representative hepatotoxic markers, also tended to increase by treatment with both agents (Table 1).

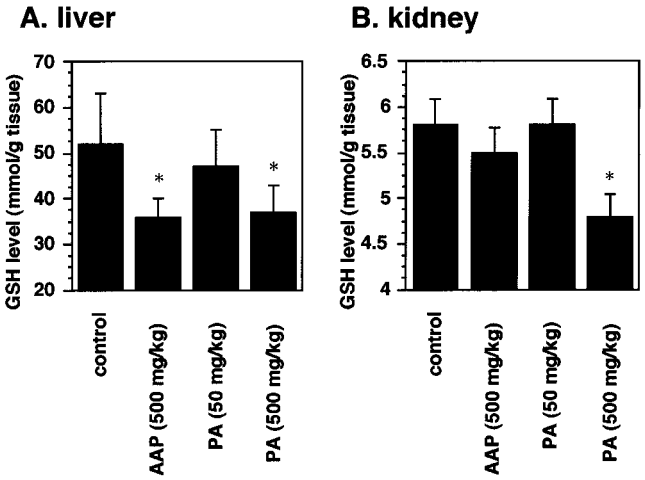

Figure 2. Effects of AAP and PA on total GSH level in ICR mouse liver (A) and kidney (B). Mice (five mice in each group) were treated with AAP (500 mg/kg) or PA (50 or 500 mg/kg). They were sacrificed 6 h after PA application, and tissue samples were removed for GSH assays. Significance: *, versus control group, $P < 0.05$.

Especially, AAP and PA application at 500 mg/kg resulted in the enhancement of AST activity by 3.1-fold ($P < 0.05$) and by 1.9-fold ($P < 0.05$), respectively, as compared with the control. Bilirubin, a degradation product of heme normally excreted in the bile from liver, was detected in the urine of all PA- and AAP-treated mice but never in the urine of the control mice (data not shown) by a commercial detection kit, Pretest 8a test paper (Wako Pure Chemical Industries). The plasmatic urea level and urinary protein level also showed a tendency to increase in mice given PA much more than in those treated with AAP. Increase in the urea level induced by PA administration was significant ($P < 0.05$). Urinary glucose, a putative marker of nephrotoxicity, was detected in PA-treated mice but never in the urine of the control mice (data not shown). These results obviously indicated the possibility of an excessive amount of PA to show a toxic effect toward mouse liver and kidney.

In the subchronic administration experiment, all animals remained healthy throughout the experimental period. The body weights and drinking water consumptions of mice did not significantly differ among the groups (data not shown). The relative weights of liver and kidney (weight/100 g of body weight) of all groups were almost comparable (Table 2). The continuous administration of PA (0.1% in drinking water) for 60 days resulted in a slight but significant enhancement of ALT activity in plasma and a decrease in GSH level in kidney (Table 2). Although the nephrotic thiobarbituric acid-reacting substance level, an overall oxidative stress marker, was slightly enhanced by relatively high doses of PA, no significant changes in other parameters concerning hepatotoxicity and nephrotoxicity were observed (data not shown). GSH depletion is known to be a marker of the presence of a thiol reactive chemical species. It is, therefore, within the range of possibility that significant attenuation of the nephortic level of GSH by a subchronic administration of high doses of PA may disturb the detoxification of other electophilic toxicants including ultimate carcinogens.

Because PA-induced hepatotoxicity is correlated with the depletion of hepatic GSH levels, we examined the negatively regulating role of GSH against hepatotoxicity

Table 1. Comparative Acute Hepato- and Nephrotoxicities of AAP and PA in ICR Mouse Liver and Kidney[a,b]

	hepatotoxicity		nephrotoxicity	
treatment	plasmatic ALT (IU/L)	plasmatic AST (IU/L)	plasmatic urea (mg/mL)	urinary protein (mg/mL)
control	22.1 ± 7.9	41.0 ± 9.8	0.20 ± 0.02	1.96 ± 1.02
AAP (500 mg/kg)	42.0 ± 9.5*	129.8 ± 36.3*	0.26 ± 0.10	1.68 ± 0.61
PA (50 mg/kg)	33.3 ± 9.0	66.3 ± 6.3*	0.21 ± 0.03	2.07 ± 1.29
PA (500 mg/kg)	36.0 ± 12.4	76.8 ± 29.0*	0.35 ± 0.10*	3.09 ± 0.87

[a] ICR mice were treated with AAP or PA (ip administration). The mice were sacrificed 6 h after AAP or PA application. The toxic parameters were determined as mentioned under Materials and Methods. [b] An asterisk indicates statistical difference from control; $P < 0.05$.

Table 2. Comparative Subchronic Hepato- and Nephrotoxicities of AAP and PA in ICR Mouse Liver and Kidney[a,b]

	hepatotoxicity			nephrotoxicity	
treatment	relative liver wt (g/100 g of body wt)	hepatic GSH (μmol/g of tissue)	plasmatic ALT (IU/L)	relative kidey wt (g/100 g of body wt)	nephrotic GSH (μmol/g of tissue)
control	4.6 ± 0.3	63.1 ± 4.5	5.6 ± 1.2	1.1 ± 0.1	13.0 ± 1.0
AAP (0.01%)	4.3 ± 0.1	55.0 ± 4.5*	8.0 ± 1.3*	1.1 ± 0.1	12.0 ± 1.2
AAP (0.1%)	4.4 ± 0.5	66.5 ± 3.6	7.0 ± 1.1	1.2 ± 0.1	14.2 ± 1.5
PA (0.01%)	4.2 ± 0.2	62.5 ± 4.6	7.0 ± 1.3	1.1 ± 0.1	11.6 ± 1.5
PA (0.1%)	4.6 ± 0.4	58.3 ± 3.3	8.0 ± 1.4*	1.2 ± 0.1	10.5 ± 0.8*

[a] ICR mice were treated with AAP or PA (in drinking water). The mice were sacrificed 60 days after the start of AAP or PA application. The toxic parameters were determined as mentioned under Materials and Methods. [b] An asterisk indicates statistical difference from control; $P < 0.05$.

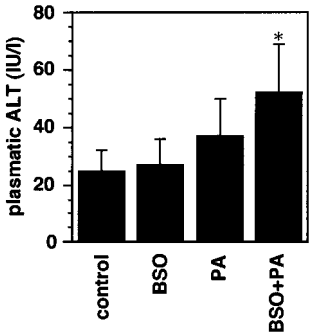

Figure 3. Effect of an inhibitor of GSH synthesis, BSO, on plasmatic ALT activity. BSO was received as an ip dose of 800 mg/kg in 0.9% NaCl 2 h before PA administration. The mice were sacrificed 6 h after PA application. The ALT activitiy was measured by using a GPT-test kit (Wako Pure Chemical Industries). Significance: *, versus PA treatment group, $P < 0.05$.

using GSH-depleted mice exposed to an inhibitor of GSH synthesis, BSO. The hepatic concentration of GSH was significantly decreased to ~50% of the control values 2 h after BSO (800 mg/kg) administration (data not shown). PA (500 mg/kg) administered 2 h after BSO induced the enhancement of ALT activity more severely than that in mice administered PA alone (Figure 3). Although statistical analysis by two-way ANOVA revealed that the treatment with PA, but not BSO, significantly affected ALT activity ($P < 0.05$, Fisher's test), interaction between the effects of PA and BSO was not observed ($P = 0.23$). Nephrotoxic parameters, including the concentration of urea in plasma, were not significantly changed (data not shown).

AAP, a widely used analgesic and antipyretic, is a safe and effective drug at therapeutic doses. AAP as a protective agent in cancer has also been described (*12*, *13*). However, overdoses of AAP can cause liver and kidney damage or even death (*14*, *15*). In the present study, we clearly demonstrated for the first time that the overdose of a naturally occurring antioxidant, PA, could cause temporary damage to the liver and kidney. Early studies on AAP hepatotoxicity indicated that liver cell injury is caused by its metabolite, *N*-acetyl-*p*-benzoquinoneimine (ABI), formed in a cytochrome P450-dependent reaction (*16*). At normal therapeutic doses, the major metabolic pathways of AAP are glucuronidation and sulfation. With an overdose of AAP, however, these pathways are saturated and the production of toxic ABI is increased, leading to a rapid depletion of the GSH level. Subsequently, ABI reacts with cellular macromolecules, causing hepatotoxicity (*17*). Although the exact mechanism of ABI-induced hepatotoxicity has not been fully elucidated, the production of ROS by redox cycling, leading to protein oxidation, and the covalent attachment with protein are suggested to be potential mechanisms (*18*). Our recent studies on the mechanism of dermatotoxicity induced by PA suggested that enhancement of tumorigenesis or contact hypersensitivity is closely correlated with the reduced GSH level in mouse skin (*8*, *9*). These indications led us to propose a hypothesis that a plausible toxic metabolite of PA, covalently binding nucleophilic residues of proteins including sulfhydryl groups or GSH, might be involved in the molecular mechanism of PA-induced hepatotoxicity. The data that the GSH-deficient mice were more sensitive to PA-induced acute damage in the liver than the control mice (Figure 3) may also support this hypothesis.

In the kidney, the administration of PA, but not AAP, significantly reduced the GSH level (Figure 2). This suggested that a different mechanism might occur for PA- and AAP-induced nephrotoxicity; PA or its metabolite(s) could additionally reduce the level of nephrotic GSH, whereas AAP or its counterpart(s) showed little effect. The GSH conjugates with redox active compounds such as *tert*-butylhydroquinone are known to be potent and selective nephrotoxicants (*10*). Therefore, GSH can be regarded as a carrier of redox-active compounds transferring to the kidney, an organ rich in γ-glutamyl transpeptidase (γ-GT). The nephrotoxicity of GSH conjugates of these compounds is dependent on the relatively high activity of γ-GT within the brush border membrane of renal proximal tubular epithelial cells. Further metabolism of the conjugates by γ-GT has been found to be a prerequisite for toxicity (*19*). The products of the reaction by γ-GT, the conjugates with cysteinylglycine dipeptide, are substrates for dipeptidases, which

similarly exist in the brush border membrane of renal proximal tubular epithelial cells. The corresponding cysteine conjugates are then transported across the brush border membrane via an amino acid transport system. Because metabolism of the phenolic compounds−GSH conjugates by γ-GT is coupled to cellular uptake, the activity of γ-GT is thus considered to be necessary for the accumulation of the conjugates into renal cells and also perhaps for the activation of the conjugates by facilitating oxidation (*20*). Although the GSH conjugate with PA has been detected but never identified (unpublished data), it is thus within the range of possibility that GSH−PA conjugate(s) may be one of the active forms that cause nephrotoxicity. The neurotoxic cysteine conjugate of dopamine, having a catechol moiety, is further metabolized to a benzothiazine derivative, which is also redox-active and can react with GSH (*21*). We speculate that further metabolism of PA−GSH conjugates in kidney may occur, and its metabolites can be thus potential nephrotoxicants. Further studies on the identification and toxic effects of not only GSH conjugates but also their metabolites are currently in progress.

In conclusion, possible toxic effects of phenolic antioxidant administration on liver and kidney were observed. Our recent study demonstrated that administration of PA at less than $^1/_{1000}$ of the toxic dose showed the most effective cancer chemopreventive activity in mouse skin (*8*). Although quantification of the amount of PA in daily consumed vegetables has not been fully evaluated, available papers reported that some kinds of vegetables or wine are significant sources of PA at concentrations of 2−10 μg/g (*22*, *23*). It is, therefore, quite difficult to achieve PA ingestion by daily food intake even at a dose of 1 mg/kg of body weight. On the other hand, much focus should be made on the safety of antioxidants administered excessively, because tablets or pills containing antioxidative vitamins or plant polyphenols, extracted and condensed from vegetables and fruits, are commonly available. Antioxidants have been considered as a double-edged sword for cancer control. Because the cancer preventive or promoting potential, threshold, and target organ of catechol antioxidants have to be distinguished in detail, further extensive study at the molecular level on the bioactivation/detoxification metabolizing mechanism of antioxidants is essential to provide supporting information.

ABBREVIATIONS USED

PA, protocatechuic acid; CyG, cyanidin glucoside; GSH, glutathione; BSO, buthionine sulfoximine; AAP, acetaminophen; ip, intraperitoneal; ALT, alanine aminotransaminase; AST, aspartate aminotransaminase; ANOVA, analysis of variance; ABI, *N*-acetyl-*p*-benzoquinoneimine; γ-GT, γ-glutamyl transpeptidase.

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