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Metabolism and Cytotoxicity of Propyl Gallate in Isolated Rat Hepatocytes: Effects of a Thiol Reductant and an Esterase Inhibitor

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SUMMARY

The relationship between the metabolism and the cytotoxic effects of propyl gallate (PG) has been studied in freshly isolated rat hepatocytes. Addition of PG (0.5–2.0 mM) to the hepatocytes elicited concentration-dependent cell death, accompanied by decreases in intracellular ATP, adenine nucleotide pools, glutathione, and protein thiols. The rapid loss of ATP preceded the onset of cell death. PG in the hepatocyte suspensions was converted to gallic acid, 4-*O*-methyl-gallic acid, and other minor products over time. In addition, PG was converted to a dimer [dipropyl-4,4′,5,5′,6,6′-hexahydroxydiphenate (PG-dimer)] and ellagic acid via autooxidation. In comparisons of the toxic effects of PG and its metabolites at concentrations of 2 mM, the parent compound PG was the most toxic. Pretreatment of hepatocytes with diazinon (100 μM), an esterase inhibitor, enhanced PG-induced cytotoxicity. This was accompanied by delay of PG loss and inhibition of gallic acid formation. The cytotoxicity of PG was also enhanced by addition of the thiol reductant dithiothreitol (4 mM), although intracellular levels of glutathione and protein thiols were maintained during the incubation period. Dithiothreitol did not affect the hydrolysis of PG to gallic acid by esterases but did delay the conversion of PG and prevented the formation of PG-dimer. In isolated hepatic mitochondria, PG elicited a concentration-dependent increase in the rate of state 4 oxygen consumption, indicating an uncoupling effect. In contrast, PG-dimer inhibited the rate of state 3 oxygen consumption. Based on the respiratory control index, the order of potency for impairment of mitochondria was PG > PG-dimer > gallic acid = 4-*O*-methyl-gallic acid = ellagic acid = propyl alcohol. These results indicate (a) that PG-induced hepatotoxicity is mediated by the parent compound and not its metabolites, (b) that toxicity is associated with ATP depletion apparently independently of cellular thiol depletion, and (c) that mitochondria may represent critical targets of PG-induced cytotoxicity.

PG (gallic acid propyl ester), as well as BHT and BHA, is used as a synthetic antioxidant in processed foods, cosmetics, and food packing materials, to prevent rancidity and spoilage. Because of its widespread usage, the potential toxicity of PG has been investigated *in vivo* and *in vitro*, to assess various toxicological properties, i.e., acute and chronic effects (1–3), carcinogenicity (4, 5), mutagenicity (6), teratogenicity (7), and cytogenetic effects (8, 9). Despite the presumed low toxicity of PG, it exerts a variety of effects on tissue and cell functions. It has been reported that PG inhibits growth of microorganisms by inhibiting respiration and nucleic acid synthesis (10), that free radicals of PG inhibit the activity of some redox enzymes (11), and that PG also inhibits hepatic microsomal hydrolase and demethylase activities (12). Furthermore, PG protects against the induction of tumors and the teratogenic effects of some chemicals (13, 14).

In general, phenolic antioxidants including PG are not easily excreted and tend to accumulate in the body (15). After oral administration of PG, >70% of the dose is absorbed in rat intestines (16, 17). Pancreatic and/or blood esterases do not hydrolyze PG (18). Consequently, gallic acid (3,4,5-trihydroxybenzoic acid), a major intermediate derived from PG by hydrolysis of the ester bond, is metabolized to 4-*O*-methyl-gallic acid by *O*-methyltransferase (16), and then both intermediates and the parent compound are conjugated as glucuronides in the liver (17). These metabolites are found in rat urine after oral administration of PG or gallic acid (16, 17, 19). Tannic acid, which is found in tea, coffee, and other plant tissues, is also metabolized to gallic acid in rats (16). Scheline (20) has reported that pyrogallol (1,2,3-trihydroxybenzene) found in rat urine may be converted from gallic acid by decarboxylation in the alimentary tract. Despite the known information about the metabolic pathway of PG and gallic acid in rats, no extensive studies have been performed on the

ABBREVIATIONS: PG, propyl gallate; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; PG-dimer, dipropyl-4,4′,5,5′,6,6′-hexahydroxydiphenate; DTT, 1,4-dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GSH, reduced glutathione; HPLC, high performance liquid chromatography; LC, liquid chromatography; MS, mass spectrometry; DMSO, dimethylsulfoxide.

relationship between the metabolism and the cytotoxicity of PG. In a previous work (21), we demonstrated that gallates, which bear long alkyl groups bound to the carboxyl group in gallic acid, are toxic to hepatocytes and that mitochondrial respiration is a common target site for the gallates. Here, we report our investigation of the action of PG and its metabolites in isolated rat hepatocytes, and we discuss the mechanism of cytotoxicity of these compounds.

Experimental Procedures

Materials. The chemical compounds used were obtained from the following companies: PG, gallic acid, and ellagic acid (purities of >98%) from the Tokyo Kasei Co. (Tokyo, Japan); 4-*O*-methyl-gallic acid (3,5-dihydroxy-4-methoxybenzoic acid) from Apin Chemicals Ltd. (Oxon, UK); GSH, adenine nucleotides, and bovine serum albumin from Sigma Chemical Co. (St. Louis, MO); diazinon [phosphothiotic acid *O*,*O*-diethyl-*O*-[6-methyl-2-(1-methylethyl)-4-pyrimidinyl] ester] (purity of >99%), DTT, and collagenase from Wako Pure Chemicals (Osaka, Japan). All other chemicals were of the highest purity commercially available.

Isolation and incubation of hepatocytes. Male Fischer 344 rats (260–290 g) were used in all experiments. Hepatocytes were isolated by collagenase perfusion of the liver, as described previously (22). Hepatocyte viability was assessed by trypan blue exclusion, and initial cell viabilities were approximately 90%.

Hepatocytes (10^6 cells/ml) were suspended in Krebs-Henseleit buffer, pH 7.4, containing 12.5 mM HEPES and 0.1% albumin. All incubations were performed in rotating, round-bottomed flasks at 37°, under a constant flow of humidified carbogen (95% O_2/5% CO_2). Reactions were started by the addition of PG or its metabolites dissolved in DMSO (final concentration, <1%). The corresponding control groups received an equivalent volume of DMSO. In some experiments using modulating agents, diazinon (100 μM) dissolved in DMSO or DTT (4 mM) dissolved in Krebs-Henseleit buffer was added to the hepatocyte suspension 15 min before the addition of PG. Aliquots of incubation mixture were taken at intervals for the determination of cell death and the concentrations of GSH, protein thiols, protein, adenine nucleotides, and PG and its metabolites.

Preparation of liver mitochondria. Liver mitochondria were isolated from male Fischer 344 rats by differential centrifugation in medium containing 0.25 M sucrose, 5 mM Tris·HCl, pH 7.4, and 1 mM EDTA (23). EDTA was omitted for the final wash and resuspension.

Measurement of respiration rates. The rate of oxygen consumption was measured polarographically with a Clark-type oxygen electrode (model 5300; Yellow Springs Instruments) at 25° in the presence (state 3) and after exhaustion (state 4) of 20 μM ADP (23). Respiration buffer (3 ml, pH 7.4) contained 0.2 M sucrose, 20 mM KCl, 3 mM $MgCl_2$, 5 mM potassium phosphate, and 1 μM rotenone. The respiration substrate was 5 mM succinate and the amount of mitochondria was 1 mg of protein/ml. The respiratory control index was calculated as the ratio of state 3/state 4 respiration.

Biochemical assays. Adenine nucleotides (ATP, ADP, and AMP) in hepatocytes were measured using HPLC, according to the procedure of Jones (24). Cellular GSH levels were determined by HPLC essentially as described by Reed *et al.* (25). Reduced protein thiol concentrations were determined by using Ellman's reagent, as described previously (26). Protein was determined by the method of Lowry *et al.* (27), using bovine serum albumin as the standard. Blebbing of hepatocytes was assayed by light microscopy and expressed as the percentage of trypan blue-excluding cells that exhibited multiple surface protrusions.

Preparation and identification of PG-dimer. After PG (20 mM) dissolved in Krebs-Henseleit buffer, pH 7.4, had been incubated for 2 hr at 37° under carbogen, the reaction mixture was subjected to preparative HPLC (TSKgel ODS-120T column, 55-mm i.d. × 300 mm, 20-μm particle size; Tosoh Co., Tokyo, Japan) for the purification of PG-dimer. The mobile phase was 50% methanol in water and the flow rate was 7 ml/min. The fraction eluting at a retention time of 15–17.5 min was collected and evaporated to dryness *in vacuo*. Structural characterization of the residue obtained from the fraction was characterized by LC/MS and ^1H and ^{13}C NMR, as follows.

Negative ion mass spectra were obtained by LC/MS with a TSQ 700 mass spectrometer (Finnigan Mat, San Jose, CA) coupled to an electrospray ionization/MS system. The HPLC system consisted of a ConstaMetric 4100 MS pump (Thermo Separation Systems, Riviera Beach, FL) and an analytical TSKgel ODS-120T column (4.6-mm i.d. × 250 mm, 5-μm particle size; Tosoh Co.). The mobile phase was 50% acetonitrile in water and the flow rate was 0.5 ml/min. LC/MS of this product showed $(M-H)^-$ at *m/z* 421.

^1H NMR and ^{13}C NMR spectra were obtained with a model JNM-A500 spectrometer (JOEL Ltd., Tokyo, Japan) operated at 500 MHz and 125 MHz, respectively. Methanol-d_4 was used as solvent and all chemical shifts (ppm) were referenced to the intrinsic value for the solvent. The ^1H NMR spectral data in parentheses are the indicated multiplicity, coupling constants (in Hz), and integration values, respectively. The assignments for ^1H were as follows: 7.13 (s, 1 H), 3.83 (t, J = 5.2, 2 H), 1.34 (q, J = 6.7, 2 H), and 0.75 (t, J = 7.3, 3 H). The assignments for ^{13}C were as follows: carboxyl carbon, 170.4 (-*C*OO-); aromatic carbon, 122.4 (C_1), 111.2 (C_2), 144.9 (C_3), 138.8 (C_4), 144.5 (C_5), and 119.5 (C_6); alkyl carbon, 68.0 (-COO-*C*H_2-), 22.5 (-*C*H_2-), and 10.7 (-*C*H_3). The chemical structure of PG-dimer is shown in Fig. 5.

Determination of PG and its metabolites by HPLC. An equal volume of chilled methanol was added to the cell suspension and then the mixture was filtered through a membrane cartridge (pore size, 0.45 μm). The eluent was injected onto an analytical TSKgel ODS-120T column (4.6-mm i.d. × 250 mm, 5-μm particle size; Tosoh Co.) equipped with a UV absorbance detector (260 nm). The mobile phases were (a) methanol/0.1 M ammonium dihydrogen phosphate (55:45, by volume, pH 5.3) and (b) methanol/0.025% phosphoric acid in water (50:50, by volume, pH 3.2), and the flow rate was 1.0 ml/min. The metabolites were identified by co-chromatography or by comparison of their HPLC retention times with those of authentic compounds in both mobile phase systems. The recoveries for PG, gallic acid, 4-*O*-methyl-gallic acid, and PG-dimer were checked by the addition of known amounts of standards to hepatocytes, and these recoveries were >85%.

Statistical analysis. Statistically significant differences between the control group and several treatment groups were made by analysis of variance, followed by Dunnett's t test.

Results

The addition of PG (0.5–2.0 mM) to isolated rat hepatocytes caused concentration-dependent acute cell death, accompanied by decreases in ATP, GSH, and protein thiol levels (Fig. 1). The appearance of surface blebs preceded the onset of cell death, and the frequency of those was correlated with the cytotoxicity. PG at 2 mM caused the abrupt depletion of cellular ATP, which was reflected by concomitant increases in the levels of ADP and AMP (data not shown) and preceded the onset of cell death. In addition, total nucleotide pools in hepatocytes treated with 2.0 mM PG were gradually depleted after a time lag of 30 min. PG did not react directly with ATP when incubated in the absence of hepatocytes (data not shown). PG also caused a concentration-dependent depletion of cellular GSH and protein thiols. GSH was rapidly depleted, before the protein thiol loss (Fig. 1, E and F).

The concentration of PG in hepatocyte suspensions decreased quickly with time, and this decrease was accompanied by the formation of gallic acid and 4-*O*-methyl-gallic acid, whose levels reached maximum concentrations after 30

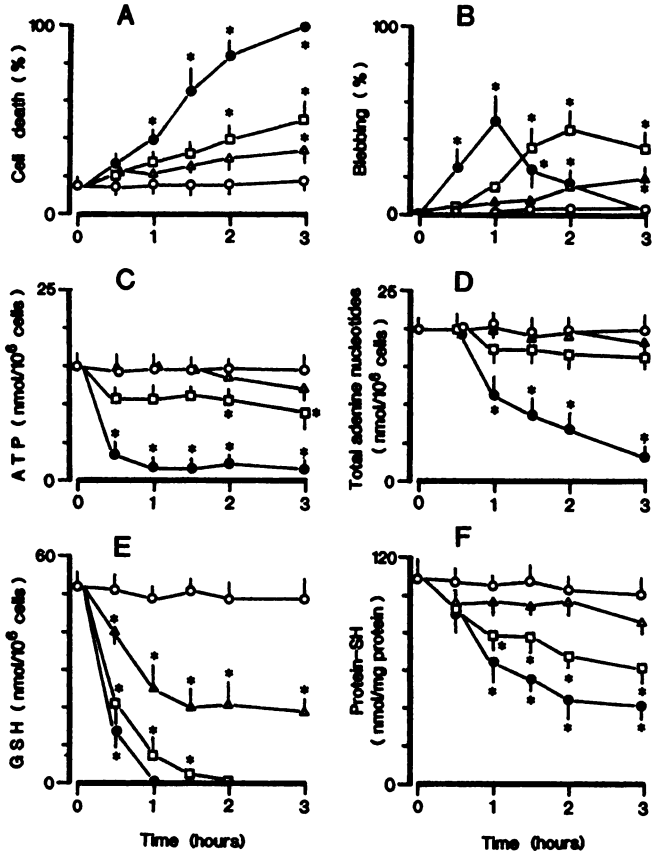

Fig. 1. Effects of PG on cell death (A), cell blebbing (B), and levels of intracellular ATP (C), total adenine nucleotides (D), GSH (E), and protein thiols (F) in isolated rat hepatocytes. Hepatocytes were incubated with no addition (○) or 0.5 mM (△), 1.0 mM (□), or 2.0 mM (●) PG. Results are expressed as the means ± standard errors of three separate experiments. *, Significant difference from values for nontreated hepatocytes ($p < 0.05$).

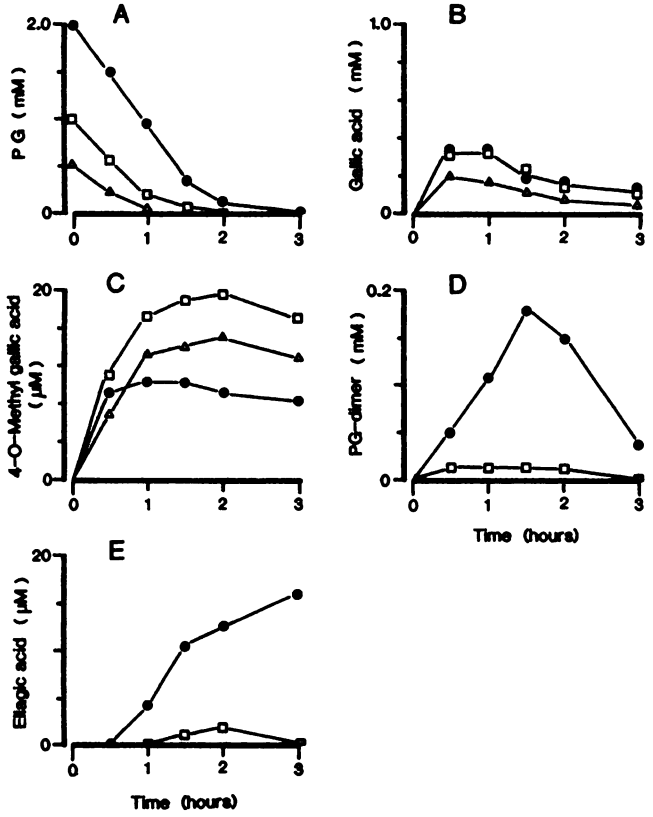

Fig. 2. Changes in the levels of PG (A) and its metabolites gallic acid (B), 4-*O*-methyl-gallic acid (C), PG-dimer (D), and ellagic acid (E) in hepatocyte suspensions. The initial concentration of PG added to hepatocyte suspensions was 0.5 mM (△), 1.0 mM (□), or 2.0 mM (●). Results are expressed as the means of two separate experiments.

and 90 min, respectively (Fig. 2). In addition, PG-dimer and ellagic acid were found in hepatocyte suspensions treated with 1 or 2 mM PG. In preliminary experiments, the formation of PG-dimer was associated with the loss of PG and the consumption of oxygen in Krebs-Henseleit buffer in the absence of hepatocytes. It is therefore apparent that PG-dimer is produced by autooxidation of PG. In this experiment, pyrogallol was not detectable in hepatocyte suspensions treated with PG during the incubation period (data not shown).

The comparative cytotoxicity of PG and its metabolites, i.e., gallic acid, 4-*O*-methyl-gallic acid, PG-dimer, ellagic acid, and propyl alcohol, was studied in isolated rat hepatocytes (Table 1). At a concentration of 2 mM, the toxicity of gallic acid, 4-*O*-methyl-gallic acid, ellagic acid, or PG-dimer in hepatocytes was less than that of PG. The decreases in intracellular ATP levels caused by these compounds were associated with the decrease in cell viability. The addition of propyl alcohol (2 mM) to cell suspensions did not affect cell viability or ATP levels during the incubation period.

Fig. 3 shows that the esterase inhibitor diazinon (100 μM) enhanced the cell death induced by PG (1 mM); the inhibitor enhanced depletion of intracellular ATP and total adenine nucleotides and accelerated the onset of surface blebbing caused by PG. In addition, diazinon enhanced losses of cellular GSH and protein thiols caused by PG (data not shown).

TABLE 1

Comparative cytotoxicity of PG and its metabolites in isolated rat hepatocytes

Hepatocytes (10^6 cells/ml) were incubated with PG and its metabolites (2 mM) in Krebs-Henseleit buffer at 37°, as described in Experimental Procedures. Cytotoxicity was determined at 90 min, as the percentage of cells taking up trypan blue. The values are expressed as the means ± standard errors of three experiments.

Treatment (2 mM)	Cell death	ATP
	%	*nmol/10⁶ cells*
None	20.3 ± 3.5	14.2 ± 2.3
PG	73.7 ± 9.8*	0.4 ± 0.2*
Gallic acid	25.5 ± 4.6	12.9 ± 2.8
4-*O*-Methyl-gallic acid	18.1 ± 3.1	13.0 ± 1.9
PG-dimer	24.9 ± 6.5	12.5 ± 3.8
Ellagic acid	21.5 ± 5.4	13.4 ± 2.1
Propyl alcohol	18.0 ± 2.8	14.1 ± 2.6

* Significant difference from values for nontreated hepatocytes ($p < 0.05$).

On the other hand, treatment with the inhibitor (100 μM) alone did not affect cell viability, intracellular ATP levels, or total adenine nucleotide pools during the incubation period. Diazinon effectively inhibited the formation of gallic acid from PG by cellular esterase and delayed the loss of PG in the hepatocyte suspensions (Fig. 3D). However, the concentration of PG-dimer was increased by pretreatment with diazinon and reached a maximum concentration at 90 min in PG-treated cell suspensions. Using HPLC analysis, we found that diazinon did not react directly with PG and that diazinon did not affect PG loss in Krebs-Henseleit buffer without hepatocytes (data not shown). These results indicate that PG,

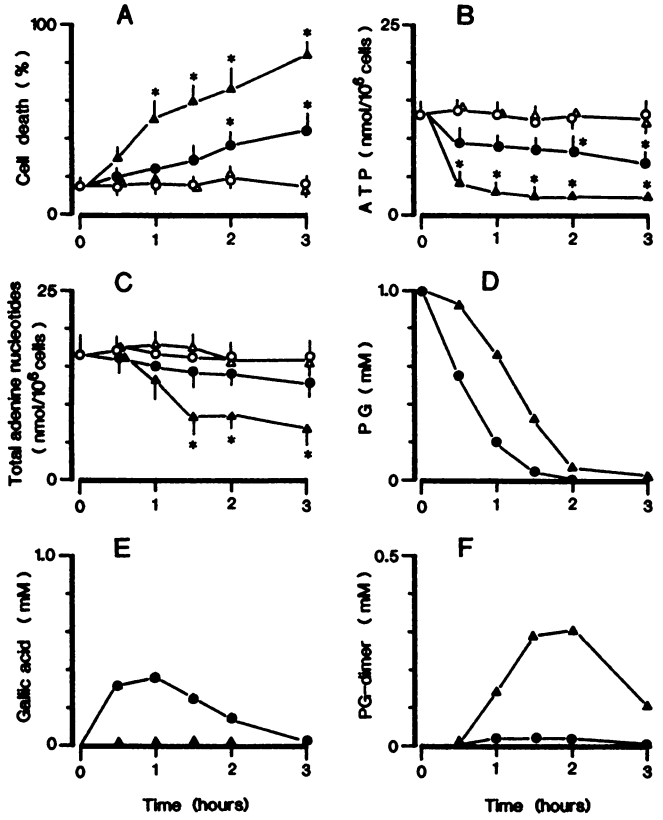

Fig. 3. Effects of the esterase inhibitor diazinon on cell death (A) and levels of intracellular ATP (B), total adenine nucleotides (C), PG (D), gallic acid (E), and PG-dimer (F) in hepatocyte suspensions treated with PG (1 mM). Hepatocytes pretreated with diazinon (100 μM) were incubated with 1.0 mM PG. ○, No addition; △, 100 μM diazinon; ●, 1.0 mM PG; ▲, 100 μM diazinon plus 1.0 mM PG. Results in A, B, and C are expressed as the means ± standard errors of three separate experiments and those in D, E, and F are expressed as the means of two experiments. *, Significant difference from values in the corresponding control group ($p < 0.05$).

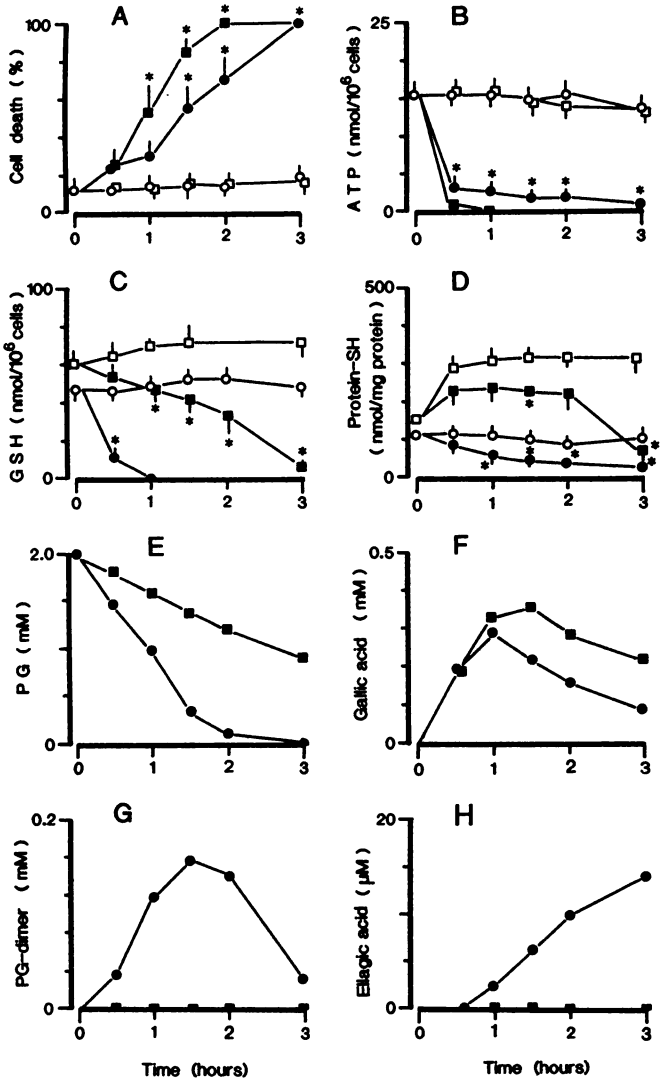

Fig. 4. Effects of the thiol reductant DTT on cell death (A) and levels of intracellular ATP (B), GSH (C), protein thiols (D), PG (E), gallic acid (F), PG-dimer (G), and ellagic acid (H) in hepatocyte suspensions treated with PG (2 mM). Hepatocytes pretreated with DTT (4 mM) were incubated with 2 mM PG. ○, No addition; □, 4 mM DTT; ●, 2 mM PG; ■, 4 mM DTT plus 2 mM PG. Results in A-D are expressed as the means ± standard errors of three separate experiments and those in E-H are expressed as the means of two experiments. *, Significant difference from values in corresponding control group ($p < 0.05$).

rather than its major metabolite gallic acid, may play an important role in the onset of cytotoxicity. The decrease in cell viability may be associated with the concentration of PG remaining in the cell suspensions.

Because it has been reported that protein thiols and cellular sulfydryl groups are important targets for reactive intermediates derived from some chemicals (28, 29), the effect of the thiol reductant DTT on PG-induced cytotoxicity was investigated (Fig. 4). Treatment of hepatocytes with DTT (4 mM) did not affect cell viability during the incubation period but did lead to elevated intracellular GSH and protein thiol levels, which were approximately 1.5- and 2.9-fold greater, respectively, than the levels of the control group. Despite this, the cytotoxicity caused by 2 mM PG was enhanced by the addition of DTT, which did not prevent the abrupt loss of intracellular ATP or adenine nucleotide pools caused by PG. Furthermore, DTT itself did not affect the formation of gallic acid derived from PG by intracellular esterase (Fig. 4F). When the loss of PG was delayed by the addition of DTT to the cell suspensions, the formation of PG-dimer and ellagic acid was prevented during the incubation period. In cell-free Krebs-Henseleit buffer, the addition of DTT (4 mM) completely prevented the loss of PG (1 mM) during a 2-hr incubation. These results indicate that the high levels of intracellular thiols produced by DTT treatment do not directly ameliorate the PG-induced cytotoxicity.

Because rapid depletion of intracellular ATP levels may be associated with inhibition of mitochondrial oxidative phosphorylation, the effects of PG and its metabolites on the oxygen consumption of isolated liver mitochondria are shown in Table 2. Addition of PG (100–500 μM) to mitochondrial suspensions caused a concentration-dependent increase in the rate of state 4 oxygen consumption, indicating partial uncoupling of mitochondrial respiration. In contrast, state 3 oxygen consumption was inhibited by PG-dimer at concentrations of 100–500 μM. Although gallic acid, 4-*O*-methylgallic acid, and propyl alcohol, at the concentrations used (250–1000 μM), did not markedly affect either state 3 or state 4 oxygen consumption, ellagic acid at 1000 μM caused a slight

TABLE 2

Effects of PG and its metabolites on mitochondrial respiration

Mitochondria (1 mg of protein/ml) were preincubated in 3 ml of respiration buffer, containing succinate (5 mM) and rotenone (1 μM), for 1.5 min at 25° (see Experimental Procedures). For the measurement of state 3 respiration, PG and its metabolites were incubated with mitochondria for 1.5 min before the addition of ADP (20 μM). Values are the means ± standard errors of three determinations.

Treatment	Mitochondrial respiration		
	State 4	State 3	RCI[a]
μM	*ng of oxygen/mg of protein/min*		
None	14.5 ± 0.5	66.0 ± 2.6	4.6
PG			
100	22.5 ± 0.8[b]	70.8 ± 2.4	3.1
250	37.6 ± 1.3[b]	66.9 ± 2.5	1.8
500	59.4 ± 3.1[b]	60.1 ± 1.9	1.0
Gallic acid			
250	14.4 ± 0.8	63.6 ± 1.9	4.4
500	15.9 ± 1.3	67.8 ± 2.4	4.3
1000	17.7 ± 0.6	67.2 ± 2.0	3.8
4-*O*-Methyl-gallic acid			
250	14.4 ± 3.0	63.2 ± 2.1	4.4
500	14.1 ± 0.9	63.2 ± 1.7	4.5
1000	15.0 ± 2.3	65.0 ± 2.9	4.3
PG-dimer			
100	13.9 ± 1.9	42.5 ± 2.0[b]	3.1
250	17.2 ± 1.9	42.0 ± 2.7[b]	2.4
500	14.7 ± 1.3	29.5 ± 1.6[b]	2.0
Ellagic acid			
250	14.0 ± 0.8	63.8 ± 2.1	4.6
500	15.1 ± 1.3	66.7 ± 2.0	4.4
1000	18.2 ± 1.4[b]	68.3 ± 1.9	3.8
Propyl alcohol			
500	14.3 ± 1.4	66.9 ± 3.4	4.7
1000	14.5 ± 2.0	65.8 ± 1.5	4.5

[a] The respiration control index (RCI) was calculated as the ratio of state 3/state 4 respiration.
[b] Significant difference from values for nontreated mitochondria ($p < 0.05$).

stimulation of state 4 oxygen consumption, without affecting state 3 oxygen consumption.

Discussion

The results obtained in the present study indicate that PG, rather than its metabolites gallic acid, 4-*O*-methyl-gallic acid, PG-dimer, ellagic acid, and propyl alcohol, is cytotoxic to isolated rat hepatocytes. PG causes a dose-dependent decrease of intracellular levels of ATP, total adenine nucleotides, protein thiols, and GSH, which consistently precedes cell death. In addition, PG elicits serious impairment of mitochondrial functions related to oxidative phosphorylation. It appears, therefore, that mitochondria are important organelle targets for PG.

Because PG is not converted to gallic acid in hepatocyte suspensions pretreated with diazinon (Fig. 3), it is apparent that the ester bond in the PG molecule is essentially stable and that PG is hydrolyzed enzymatically, by cellular carboxylesterases, to gallic acid and propyl alcohol. Hepatic carboxylesterases (EC 3.1.1.1.) are a group of β-esterases that hydrolyze a wide range of xenobiotic ester substrates. When carboxylesterase activity is inhibited by diazinon, PG is converted mainly to PG-dimer, without gallic acid, via autooxidation. This result indicates that the PG-dimer is not derived from PG via gallic acid. Indeed, gallic acid added to Krebs-Henseleit buffer did not yield PG-dimer during a 3-hr incubation (data not shown). In preliminary experiments, we found that the conversion of PG to PG-dimer was accompanied by the consumption of oxygen in Krebs-Henseleit buffer without hepatocytes. Oxygen was then regenerated by the addition of catalase (10 units/ml) and superoxide dismutase (10 units/ml) to the buffer at the end of the reaction (data not shown). This reaction indirectly indicates that superoxide anion radical is produced by the autooxidation of PG. PG metabolism, including autooxidation, in hepatocyte suspensions did not induce the accumulation of cellular malondialdehyde, an index of lipid peroxidation, during the incubation period (data not shown). The formation of PG-dimer in hepatocyte suspensions is not directly involved in the onset of cell death, because (a) PG-dimer was present in trace amounts in cell suspensions treated with DTT during the incubation period (Fig. 4G) and (b) PG-dimer itself added to hepatocyte or mitochondria suspensions was less toxic than its parent compound, PG (Tables 1 and 2). The delay of PG loss produced by addition of diazinon enhanced the cytotoxicity of PG, indicating that the toxicity is directly due to the parent compound, rather than its metabolites, and that metabolism by the esterase, as well as glucuronidation (17), plays an important role in the detoxification of PG. It is well known that ellagic acid possesses antimutagenic and anticarcinogenic activities. A small amount (approximately 15 μM) of ellagic acid was found in hepatocyte suspensions after a lag time of approximately 30 min (Fig. 2). In hepatocyte suspensions that had been boiled for 10 min, PG was converted to PG-dimer and small amount of ellagic acid during the incubation period (data not shown). In addition, ellagic acid was found in rat serum and liver after oral administration of PG.[1] Hathway (30) has reported that no ellagic acid is produced from gallic acid via autooxidation and that gallate ester dimerization precedes hydrolysis of the ester group in the autooxidation. On the basis of the present and previous studies, Fig. 5 shows a schematic outline of the relationship between PG metabolites and cytotoxic effects in isolated rat hepatocytes.

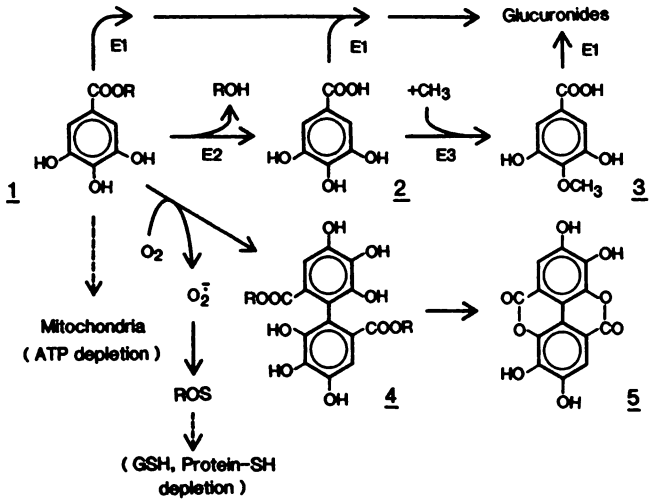

Fig. 5. Proposed mechanism of cytotoxic effects induced by PG and its metabolites in isolated rat hepatocytes. *Solid arrows*, metabolic routes; *dotted arrows*, effects of PG and its metabolites in hepatocytes. *E1*, glucuronidation; *E2*, carboxyl ester hydrolysis; *E3*, *O*-methylation; *ROS*, reactive oxygen species; *R*, propyl group; *1*, PG; *2*, gallic acid; *3*, 4-*O*-methyl-gallic acid; *4*, PG-dimer; *5*, ellagic acid.

The addition of PG to hepatocyte suspensions led to rapid

[1] Y. Nakagawa, unpublished observations.

depletion of intracellular GSH and protein thiols (Fig. 1). It is well known that the sulfydryl group in proteins and nonproteins is involved in the maintenance of various cellular functions, including the enzymatic activities of thiol-dependent enzymes (31). For example, Vartanyan *et al.* (32) reported that inactivation of lactic dehydrogenase by PG was due to the oxidation of enzyme sulfydryl groups by PG radicals. Agatova and Emanuel (33) found that radicals of PG accelerated the conversion of enzyme sulfydryl groups to disulfide bonds during oxidation. The depletion of protein thiols after GSH loss in hepatocytes correlates with the onset of cytotoxicity induced by some chemicals (34, 35). We have reported that the modification of protein thiols by oxidative stress or arylation is assumed to be a major factor in the cytotoxicity induced by diquat, hydroxyl biphenyls, and other chemicals (36, 37). DTT is a potential reductant of oxidized sulfydryl groups in cellular proteins; protein thiol levels were increased approximately 2.5-fold by the supplementation of 4 mM DTT after 90 min (Fig. 4). In addition, a slight elevation of GSH levels was observed in untreated hepatocytes after DTT supplementation. The origin of this GSH may be related to reduction of oxidized glutathione, release from glutathione-protein mixed disulfides, and *de novo* synthesis of GSH in hepatocytes. In this study, although the supplementation of hepatocytes with DTT facilitated the maintenance of high levels of cellular GSH and protein thiols during the incubation period, PG-induced cytotoxicity was not effectively ameliorated by DTT, which delayed PG loss in hepatocyte suspensions (Fig. 4). DTT did not affect the hydrolysis of PG by cellular esterases (Fig. 4) but effectively inhibited the formation of PG-dimer via oxidation reactions. Similarly, because DTT protects PG from spontaneous oxidation in Krebs-Henseleit buffer without hepatocytes (data not shown), it seems that DTT prevents the formation of phenoxyl radicals derived from PG during oxidation. Thus, the formation of PG-dimer and the loss of protein thiols are not directly involved in the onset of PG-induced cytotoxicity. However, the loss of protein thiols at a later stage may represent a proximate event indirectly contributing to PG-induced cytotoxicity. Based on these results, PG seems to be the ultimate hepatotoxicant mediating toxicity independently of its activated intermediates, which are presumed to be PG radicals generated during the formation of the PG-dimer by autooxidation.

Mitochondrial dysfunction and consequent disruption of ATP production are common mechanisms for hypoxia and toxic chemicals causing acute cytotoxicity (38–40). Because (a) the depletion of ATP caused by PG alone was faster than that of protein thiols (Fig. 1) and (b) DTT did not affect the abrupt decrease of cellular ATP levels caused by PG (Fig. 4), it appears that an irreversible loss of cellular ATP plays an important role in the onset of PG-induced cytotoxicity in the period immediately after exposure to PG. The depletion of intracellular ATP and total adenine nucleotide pools caused by PG is closely associated with impairment of mitochondrial respiration, because mitochondria are the main site of ATP synthesis via oxidative phosphorylation. The increase in state 4 respiration caused by PG indicates uncoupling of oxidative phosphorylation in mitochondrial respiration (Table 2). Based on the respiration control index, a sensitive parameter of mitochondrial dysfunction, it follows that the order of potency is PG > PG-dimer > gallic acid = 4-*O*-methyl-gallic acid = ellagic acid = propyl alcohol. Thus, PG itself has an inhibitory action on mitochondrial function. We demonstrated that an abrupt loss of ATP, with a concomitant increase in cellular ADP and AMP, consistently preceded cell death (21). BHA and other phenolic compounds are effective inhibitors of a number of FAD- and NAD^+-containing oxidases and dehydrogenases, via reaction mechanisms that exhibit complex kinetics (41, 42). Law *et al.* (43) have shown that the lipophilic antioxidant BHT affects the interior hydrophobic portion of the membrane more than the polar hydrophilic phase. The cytotoxicity caused by BHA or BHT is thought to depend on inhibition of mitochondrial bioenergetics via the perturbation of membrane function induced by both compounds (44). Based on these results, it seems that the phenolic hydroxy groups in PG are essential for the inhibition of mitochondrial respiration and that the alkyl group esterified to gallic acid enhances the cytotoxicity of gallic acid.

The formation of plasma membrane blebbing is recognized as an early effect of toxic and anoxic damage in hepatocytes (39). Depletion of cellular ATP may be related to the onset of plasma membrane damage, such as blebbing of the cell membrane, because it has been reported that the maintenance of ATP levels is important for polymerization of microfilaments and microtubules (45). In addition, the formation of plasma membrane blebbing has been attributed to collapse of the mitochondrial membrane potential and elevation of cytosolic free Ca^{2+}, as well as alkylation or oxidation of sulfydryl groups on cytoskeletal proteins (45). In preliminary experiments, PG (500 μM) caused the release of Ca^{2+} from isolated rat mitochondria (data not shown). Although the appearance of plasma membrane blebbing precedes the onset of cell death induced by PG (Fig. 1), the appearance of blebbing is prevented by supplementation with DTT (data not shown). Therefore, the potency of cytotoxicity caused by PG may depend on (a) the initial concentration of PG to which hepatocytes are exposed, (b) the concentration of PG remaining in cell suspensions during the incubation, and (c) the rate of cellular ATP depletion, possibly due to mitochondrial dysfunction.

In conclusion, this study has demonstrated that PG, rather than its metabolites, causes acute cytotoxicity in isolated rat hepatocytes. Hepatotoxicity is enhanced when the metabolism of PG is inhibited by diazinon, a carboxylesterase inhibitor, or DTT, a thiol reductant. PG-induced hepatotoxicity is preceded by intracellular ATP depletion, independently of protein thiol loss. The finding that isolated mitochondrial respiration was uncoupled in the presence of PG suggests that impairment of mitochondria, with decreased ATP synthesis, may the mechanism of PG hepatotoxicity.

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