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Activation of rat liver microsomal glutathione *S*-transferase by gallic acid

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Abstract

The effect of phenolic antioxidants on the rat liver microsomal glutathione *S*-transferase (MGST1) was investigated in vitro. When microsomes were incubated with various polyphenolic antioxidants, gallic acid (3,4,5-trihydroxybenzoic acid) markedly increased MGST1 activity and the increase was prevented in the presence of superoxide dismutase (SOD) or catalase. The MGST1 activity increased by gallic acid was decreased by further incubation with sodium arsenite, a sulfenic acid reducing agent, but was not with dithiothreitol, a disulfide bond reducing agent. The incubation of microsomes with gallic acid in the presence of the NADPH generating system which generates reactive oxygen species (ROS) through cytochrome P-450 system increased the MGST1activity in spite of scavenging the ROS and the increase was also depressed by SOD/catalase. The increase of MGST1 activity by gallic acid was prevented by co-incubation with a stable radical, 1,1-diphenyl-2-picrylhydrazyl or ferric chloride. These results suggest that the gallic acid acts as a pro-oxidant and activates MGST1 through oxidative modification of the enzyme.

Keywords: Glutathione *S*-transferase; Gallic acid; Reactive oxygen species; Antioxidant; Liver microsome

Introduction

Glutathione *S*-transferases (GST; EC 2.5.1.18) are phase II drug-metabolizing enzymes responsible for the glutathione conjugation of a variety of xenobiotics such as carcinogens, therapeutic drugs and highly reactive lipid peroxidation products (Wilice and Parker, 1994; Andersson et al., 1994). The activity or content of GSTs in cells, therefore, affects the chemical toxicity of various compounds or drug resistance of chemotherapeutic agents (Hayes and Pulford, 1995). Mammalian GSTs involve cytosolic and membrane-bound forms which are encoded by different gene families (Mannervik, 1985). It has been evidenced that cytosolic GST are induced or inhibited by naturally occurring products (Zhang and Das, 1994; Fiander and Schneider, 2000; Chou et al., 2000), however the effects of these compounds on microsomal GST are not studied well. Rat liver microsomal GST (MGST1) is a homotrimer which contains one cysteine residue per subunit protein and comprises 3% of the microsomal protein (Andersson et al., 1994; Morgenstern et al., 1985). MGST1 activity is low in native form and is activated by various methods including reversible and irreversible modifications of the SH group (Morgenstern et al., 1979; Aniya and Anders, 1989a). MGST1 is also activated with reactive oxygen or nitrogen species by forming a mixed disulfide bond, protein dimmer or nitration (Aniya and Anders, 1992; Aniya and Naito, 1993; Aniya et al., 2001; Ji and Bennett, 2003). Since MGST1 can detoxify toxic metabolites from membrane lipid peroxidation, it has been suggested that the activated MGST1 is protective against membrane damages caused by oxidative stress (Mosialou et al., 1993).

Recently various actions including biological, pharmacological and medical properties of naturally occurring polyphenolic antioxidants have been extensively investigated (Chung et al., 1998; Knight, 2000; Tsuda et al., 2004). Increasing intake of herbal medicines, healthy foods, or supplements of natural products including polyphenols prompts us to study an interaction of antioxidants with drug metabolizing enzymes and we reported the inhibitory action of herbal antioxidants to these enzymes (Gyamfi et al., 2000; Gyamfi et al., 2004).

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In this study the effect of polyphenolic antioxidants involved in medicinal herbs and related synthetic compounds commercially available on MSGT1 activity was examined in vitro and the activation of MGST1 by gallic acid was investigated in detail.

Materials and methods

Chemicals

Reduced glutathione (GSH), glucose 6-phosphate, β-nicotinamide adenine dinucleotide phosphate (NADP), dithiothreitol (DTT), quercetin, catechin, caffeic acid, tannic acid, pyrogallol, ellagic acid, *n*-propyl gallate, catalase and superoxide dismutase (SOD) were obtained from Sigma Chemicals (St. Louis, MO, USA). Gallic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 1-chloro-2,4-dinitrobenzene (CDNB) were from Wako Pure Chemicals (Osaka, Japan). Glucose 6-phosphate dehydrogenase was obtained from Oriental Yeast (Tokyo, Japan). 5,5′-Dimethyl-1-pyrroline *N*-oxide (DMPO) was from Dojindo Laboratories (Kumamoto, Japan). All reagents used were of analytical grade.

Preparation of liver microsomes

Male Sprague–Dawley rats (300–400 g) from Nihon SLC Co. (Shizuoka, Japan) were used. After overnight starvation rats were killed by decapitation and the liver was removed after perfusion with ice-cold 1.15% potassium chloride solution followed by homogenization with the same solution. Microsomes were prepared by differential centrifugations as described previously (Gyamfi et al., 2000). One washed microsomes thus obtained were stored at − 80 °C and used within 3 days. The microsomes were used in suspension (6–9 mg protein/ml) with 0.05 M potassium phosphate buffer (pH 7.4). Protein concentration in microsomes was determined by the method of Lowry et al. (1951).

Effect of antioxidants on MGST1 activity

Microsomes were incubated with antioxidants in 0.05 M potassium phosphate buffer (pH 7.4) in total volume of 1 ml at room temperature (25 °C) or at 30 °C for appropriate times and then aliquot (200 μl) was used for GST assay. Purified MGST1 (25 μl) was also incubated with gallic acid in total volume of 200 μl at 30 °C for 5 min followed by measuring MGST1 activity as well. In the case the glutathione involved in the purified MGST1 preparation was removed through Sephadex G-25 column just before the experiment. MGST1 activity was measured using 1 mM CDNB and 5 mM GSH as substrates by the method of Habig et al. (1974) at 25 °C.

Measurement of radical scavenging activity by using chemiluminescence (CL) analyzer

The effect of antioxidants on cytochrome P-450-derived CL production was measured with a CL detector (CLD-110, Tohoku Electric Co., Sendai, Japan). Microsomes were incubated with or without antioxidants (0.1 ml) in the presence of 0.2 ml of the NADPH generating system (NADP 0.33 mM, G6P 8 mM, G6P dehydrogenase 0.5 U, $MgCl_2$ 6 mM) in 0.05 M phosphate buffer (pH 7.4) in total volume of 1 ml and the CL was counted at 30 °C for 5 min. At 0 and 5 min, 200 μl of the reaction mixture were taken for measuring MGST1 activity.

Effect of various agents on MGST1 activation by gallic acid

Microsomes were incubated with 1 mM gallic acid in 0.05 M phosphate buffer (pH 7.4) in the absence or presence of SOD (750 U/ml) or catalase (12 600 U/ml) in total volume of 1 ml at 25 or 30 °C and then MGST1 activity was measured at the indicated times.

The effect of reducing agents was examined as follows: microsomes were incubated with 1 mM gallic acid in 0.05 M phosphate buffer (pH 7.4) at 30 °C for 5 min in total volume of 200 μl and then 5 μl of reducing agents (DTT or sodium arsenite, final concentration of 10 mM) were added and further incubated at room temperature for 30 min. MGST1 activity was then measured at indicated times.

To confirm DPPH or ferric chloride effects, the microsomes were mixed with 1 mM of DPPH and ferric chloride followed by addition of gallic acid (1 mM) in 0.05 M phosphate buffer (pH 7.4) and the MGST1 activity was measured after the incubation at 30 °C for 5 min.

Enzyme kinetics

Microsomes were incubated with various concentrations of GSH or CDNB in the absence or presence of gallic acid (1 mM) in 0.05 M phosphate buffer (pH 7.4) at 30 °C for 5 min and then MGST1 activity was measured. When CDNB was varied the GSH concentration was kept at 5 mM and vice versa (1 mM CDNB).

Measurement of gallic acid radical

The intensity of gallic acid radical was determined with electron spin resonance (ESR) spectroscopy (JES-FR30, JOEL, Tokyo). Gallic acid (1 and 5 mM) was resolved in 0.1 M Tris–HCl buffer (pH 7.4, 8.0, 8.5) or 0.5 N NaOH solution in total volume of 200 μl and then ESR spectrum was recorded at room temperature after 40 s. Instrument settings were as follows: center field; 336.2 mT, microwave power; 4 mW, amplitude; 500, modulation amplitude; 0.2 mT, time constant; 0.1 s, and the sweep time; 0.5 min.

Results

Effects of antioxidants on MGST1 activity

The effect of various antioxidants on MGST1 activity was measured at 1 mM (Fig. 1). Gallic acid and caffeic acid increased MGST1 activity markedly (571% of control) and slightly (145%) respectively whereas quercetin, tannic acid, ellagic acid and chlorogenic acid decreased the activity to 0%, 15%, 5%, and 42%,

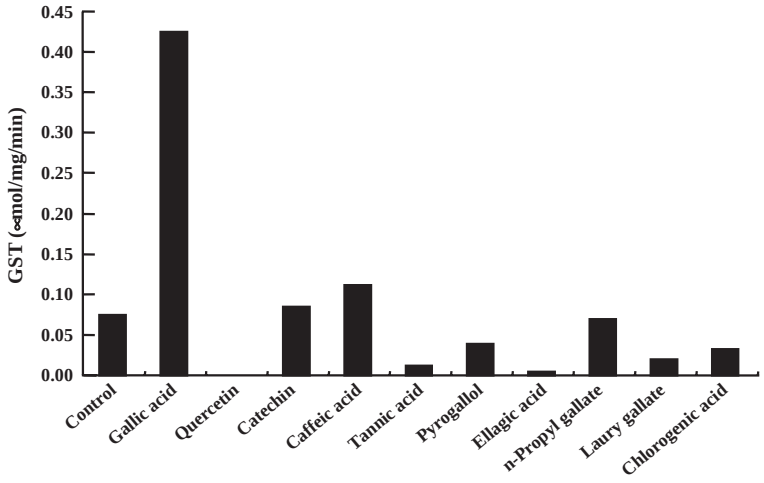

Fig. 1. Effect of phenolic compounds on MGST1 activity. Microsomes were incubated with various phenolic compounds (1 mM) in 0.05 M phosphate buffer (pH 7.4) at 30 °C for 5 min and then GST activity was measured as described in Materials and methods. Each column shows mean for triplicate determinations.

respectively. Gallic acid derivatives such as propyl gallate, lauryl gallate and pyrogallol did not increase the MGST1 activity.

Antioxidant properties of gallic acid

Fig. 2 shows the effect of gallic acid on chemiluminescence (CL) production in microsomes in the presence of the NADPH generating system (g. s). CL production from the microsomes was increased gradually and reached to 500 counts at 5 min whereas it was suppressed by gallic acid, suggesting that gallic acid at 1 mM acts as an antioxidant. In the same conditions MGST1 activity was 0.059 μmol/mg/min at 0 min and increased to 278% by the NADPH g. s plus gallic acid (1 mM) at 5 min although the activity was increased to 132% by the NADPH g. s alone. These results clearly show that gallic acid depresses CL production and activates MGST1.

Dose dependent effect and kinetic studies of gallic acid for MGST1 activity

When microsomes were incubated with various concentrations of gallic acid, MGST1 activity was increased dose dependently and reached almost plateau levels at 1 mM (840% of control) while pyrogallol and caffeic acid showed small increase of the GST activity (160% and 113% at 1 mM, respectively) followed by a slight decrease at higher concentrations (Fig. 3). In kinetic studies MGST1 activity was increased either when GSH was the variable substrate and CDNB was the fixed substrate or vice versa in the presence of gallic acid (Fig. 4). V_{max} values for GSH were 0.064 μmol/mg/min in control and 0.500 μmol/mg/min by gallic acid and for CDNB 0.067 μmol/mg/min in control and 0.238 μmol/mg/min by gallic acid. K_m values for GSH were 0.156 mM in control and 12.5 mM in gallic acid and for CDNB 0.073 mM in control and 0.23 mM by gallic acid.

Effect of superoxide dismutase (SOD) and catalase on gallic acid-induced activation of MGST1

Fig. 5 shows the time course effect of gallic acid on MGST1 activity in the presence or absence of SOD or catalase at 30 °C (A) and at room temperature (25 °C) (B). When microsomes were incubated with 1 mM gallic acid at 30 °C, MGST1 activity was increased to 648% at 5 min,

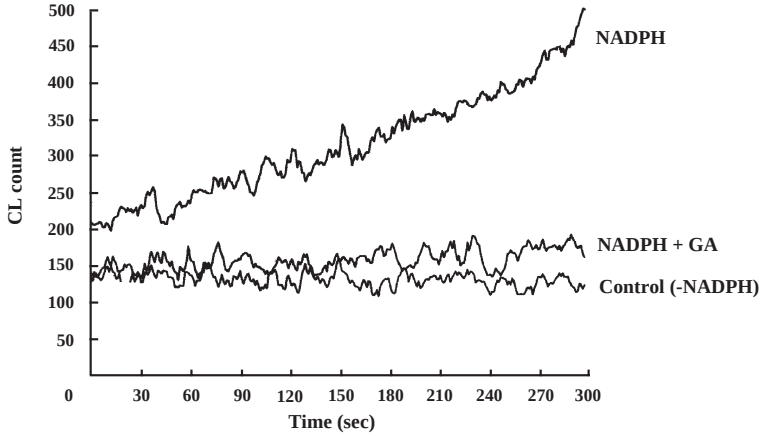

Fig. 2. Effect of gallic acid (GA) on chemiluminescence (CL) production. Microsomes were incubated with or without gallic acid (1 mM) in the presence or absence of the NADPH generating system in 0.05 M phosphate buffer (pH 7.4) and then CL production was recorded at 37 °C.

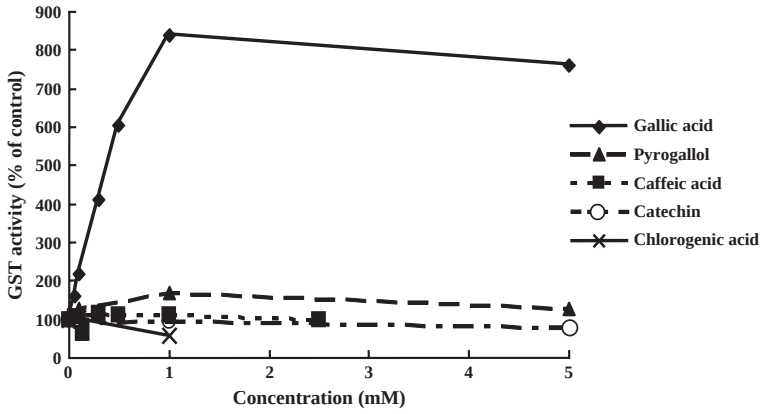

Fig. 3. Concentration-dependant effect of phenolic compounds on MGST1 activity. Microsomes were incubated with indicated concentrations of phenolic compounds in 0.05 M phosphate buffer (pH 7.4) at 30 °C for 5 min and then GST activity was measured. MGST1 activity in control was 0.07 μmol/mg/min. Each point shows mean of duplicate incubations.

and then gradually decreased and reached the control level at 20 min. On the other hand in the presence of SOD and catalase the MGST1 activity at 5 min was 427% and 271% of control, respectively, and gradually increased and reached to 755% and 538% at 20 min. In the case of the incubation at room temperature (25 °C) MGST1 activity was increased by gallic acid to 589% at 5 min, to 826% at 10 min and was still high at 20 min showing 563% of control. The increase in MGST1 activity by gallic acid was deceased in the presence of SOD to 261% at 5 min and to 398% at 10 min and reached to the same level as that of gallic acid alone at 20 min. Thus it was demonstrated that the activation of MGST1 by gallic acid was decreased by SOD and catalase.

Table 1 indicates the effect of SOD and catalase on gallic acid-induced MGST1 activation in the presence or absence of the NADPH generating system. Similarly the increase in MGST1 activity by gallic acid was prevented by SOD and catalase. Since SOD converts superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2) which is decomposed to oxygen and water by catalase, these data suggest that O_2^- and H_2O_2 are involved in the MGST1 activation by gallic acid.

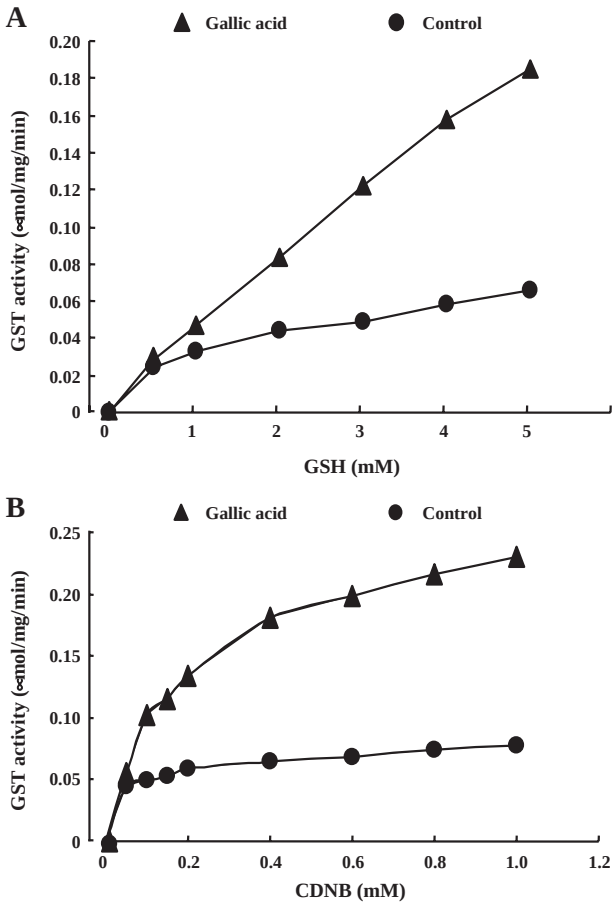

Fig. 4. Effect of substrate concentrations on gallic acid-induced activation of MGST1. Microsomes were incubated with gallic acid (▲; 1 mM) or without (control; ●) at 30 °C for 5 min and then GST activity was measured with various concentrations of GSH and 1 mM CDNB (A) or with various concentrations of CDNB and 5 mM GSH (B). Each point shows mean for duplicate incubations.

Effect of reducing agents and radical scavengers on gallic acid-induced activation of MGST1

As shown in Fig. 6, the MGST1 activity in the microsomes was increased time-dependently by incubation with 1 mM gallic acid at 25 °C (573% of the starting level at 5 min, 1038% at 15 min and 823% at 30 min). When DTT was added to the reaction mixture at 5 min after the incubation with 1 mM gallic acid, the MGST1 activity increased to 510% by gallic acid was kept almost at the same level until 30 min (542% at 30 min). Thus it is clear that the time dependant increase in MGST1 activity by gallic acid was prevented by DTT, a disulfide bond reducing agent. On the contrary the MGST1 activity was decreased to 280% at 15 min and 180% at 30 min by adding a sulfenic acid reducing agent, sodium arsenite, suggesting the oxidation of the SH of MGST1 to sulfenic acid (SOH) by the gallic acid.

We examined the effect of ferric chloride and DPPH in gallic acid induced activation of MGST1. The increase in MGST1 activity (515% of control) by gallic acid was completely prevented in the presence of Fe^{3+} or DPPH (Fig. 7).

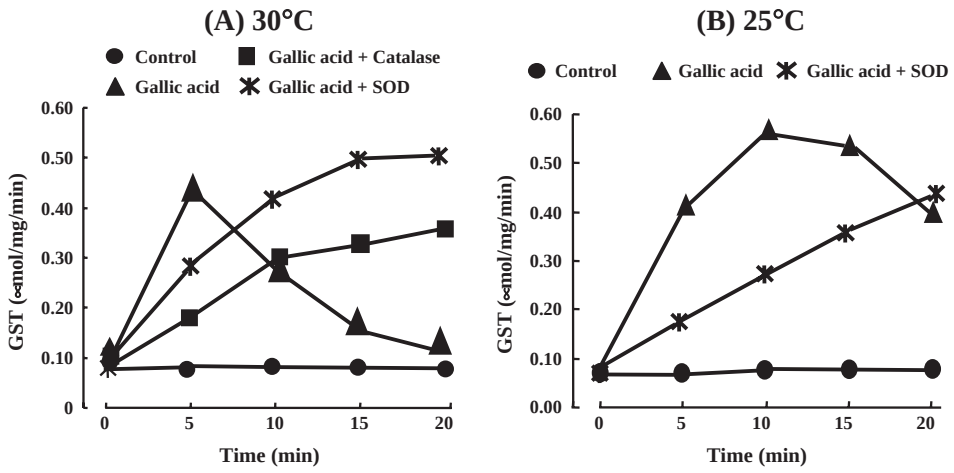

Fig. 5. Effect of SOD and catalase on gallic acid-induced activation of MGST1. Microsomes were incubated with gallic acid (1 mM) in the presence or absence of SOD (750 U/ml) or catalase (12 600 U/ml) at 30 °C (A) and room temperature (25 °C) (B) and then GST activity was measured at indicated times. Each point shows mean of duplicate incubations.

Gallic acid radical formation

As shown in Fig. 8, gallic acid radical was scarcely detected in 0.1 M phosphate buffer (pH 7.4) by ESR spectrometer and the intensity of the radical was increased according to the increase of pH. In 0.1 N NaOH solution more gallic acid radical was observed and the life span of the radical was 30 min. Superoxide anion radical was not detected in ESR spectrometer when gallic acid was mixed with DMPO in 0.05 M phosphate buffer (pH 7.4).

Effect of gallic acid on purified MGST1 activity

Purified MGST1 activity was increased dose-dependently by incubation with various concentrations of gallic acid and reached almost plateau at 0.5 mM (850% of control) (Fig. 9).

Discussion

It was clarified in this study that among various polyphenolic antioxidants gallic acid markedly increased the MGST1 activity. Gallic acid is known as a natural antioxidant involved in diets, teas or medicinal herbs with both inhibitory and scavenging actions of reactive oxygen species (ROS) (Stupans et al., 2002; Aniya et al., 2002; Pannala et al., 1997). On the other hand gallic acid is known to generate ROS or to induce apoptosis in cancer cell lines through ROS including hydrogen peroxide (Inoue et al., 2000; Inoue et al., 1994; Sakagami et al., 1997; Nakagawa et al., 1995), showing that gallic acid can act as an antioxidant and a pro-oxidant. In considering that MGST1 contains one cysteine residue per subunit and is activated by ROS through the oxidative modification of the cysteine thiol (Aniya and Anders, 1992; Aniya and Naito, 1993; Aniya and Anders, 1989b), it could be postulated that ROS generated from gallic acid causes an activation of the MGST1. To confirm the possibility antioxidant enzymes were tested. When rat liver microsomes were incubated with gallic acid in the presence of SOD or catalase, the increase in the MGST1 activity by gallic acid was depressed (Fig. 5). It was therefore suggested that superoxide

Table 1
Effect of SOD or catalase on gallic acid induced MGST1 activation

Treatment	NADPH	GST activity (μmol/mg/min)	Percent
Control	–	0.0594	100
	+	0.0782	132
Gallic acid	–	0.3431	578
	+	0.1649	278
Gallic acid+SOD	–	0.1743	293
	+	0.0735	134
Gallic acid+catalase	–	0.1500	253
	+	0.0750	126

Microsomes (0.7–0.9 mg/ml) were incubated with or without gallic acid in the presence or absence of the NADPH generating system in 0.05 M phosphate buffer (pH 7.4) at 30 °C for 5 min and GST activity was measured.

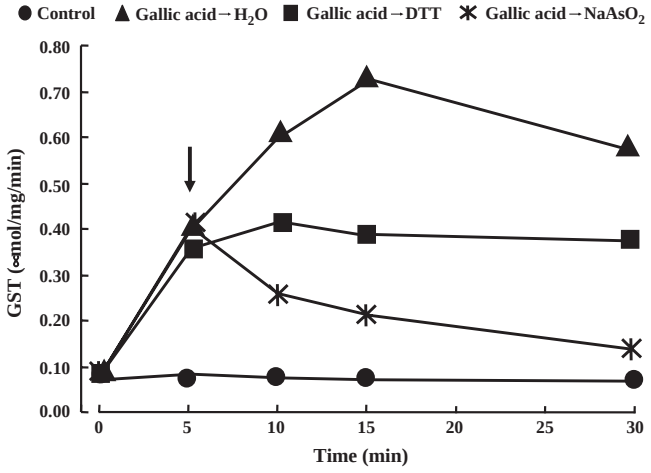

Fig. 6. Effect of dithiothreitol and sodium arsenite on gallic acid-induced MGST1 activation. Microsomes were incubated in 0.05 M phosphate buffer (pH 7.4) without or with gallic acid (1 mM) at 30 °C and then DTT (10 mM) or sodium arsenite ($NaAsO_2$, 10 mM) was added to the incubation mixture at 5 min followed by further incubation at room temperature. GST activity was measured at the indicated times. Each point shows mean for duplicate determinations.

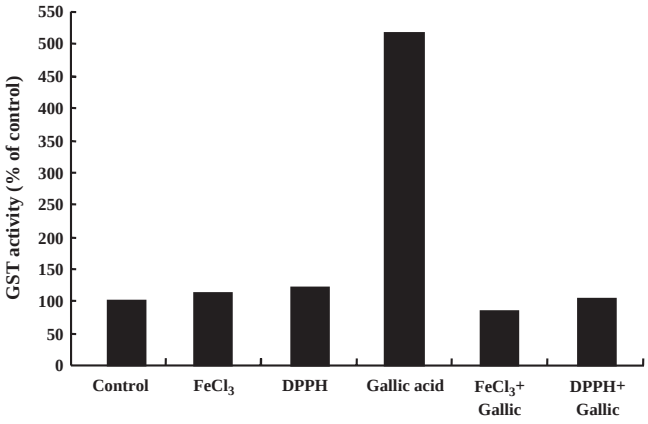

Fig. 7. Effect of ferric chloride and DPPH on gallic acid-induced activation of MGST1. Microsomes were incubated with gallic acid (1 mM) in the absence or presence of ferric chloride ($FeCl_3$, 1 mM) or DPPH (1 mM) in 0.05 M phosphate buffer (pH 7.4) at 30 °C for 5 min and then GST activity was measured. GST activity of control was 0.085 μmol/mg/min. Each column shows mean for duplicate incubations.

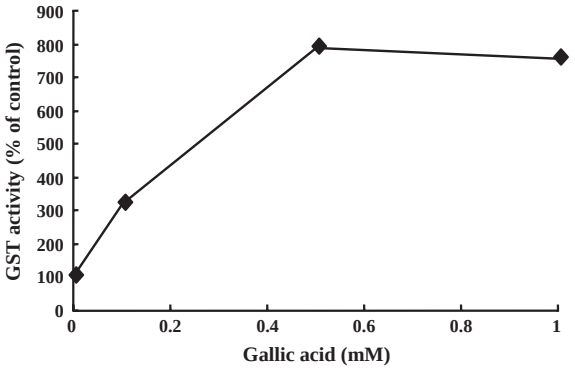

Fig. 9. Effect of gallic acid on purified MGST1 activity. GSH-free purified MGST1 was incubated with various concentrations of gallic acid at 30 °C for 5 min in 0.05 M potassium phosphate buffer (pH 7.4) in total volume of 200 μl and then GST activity was measured. Each point indicates mean of duplicate incubations. GST activity in control was 0.704 μmol /mg/min.

anion/hydrogen peroxide is involved in the activation of MGST1 by gallic acid. However we could not detect superoxide anion in the reaction mixture by using ESR spectroscopy (data not shown). Thus it was assumed that the gallic acid may produce superoxide by giving one electron to molecular oxygen in the reaction solution by its reducing action, with this superoxide anion instantly forming H_2O_2 by dismutation reaction as described (Sakagami et al., 2001). It was therefore suggested that the gallic acid produces ROS by auto-oxidation with atmospheric oxygen, consequently the ROS activates MGST1. This activation mechanism can be deduced from the observations that gallic acid-derived activation of MGST1 was reduced in hypoxic conditions (data not shown).

As a ROS-mediated activation of MGST1 by gallic acid a dimer formation of MGST1 via disulfide bond was assumed as seen by H_2O_2 treatment (Aniya and Anders, 1992). However, in Western blot analysis using anti-MGST1 antibody we could not detect a MGST1 dimer after the treatment of microsomes with gallic acid (data not shown). In addition when a disulfide bond reducing agent, dithiothreitol (DTT) was added to the incubation mixture at 5 min after the treatment with gallic acid, the MGST1 activity was kept almost at the same level until 30 min whereas MGST1 activity was increased time-dependently by gallic acid alone (Fig. 6). Thus it was demonstrated that further increase in MGST1 activity from 5 to 30 min by gallic acid was prevented by DTT but once increased MGST1 activity (by 5 min) was not reduced by DTT. This means that the MGST1 activation by gallic acid is not due to a disulfide bond formation of the enzyme. We examined the effect of

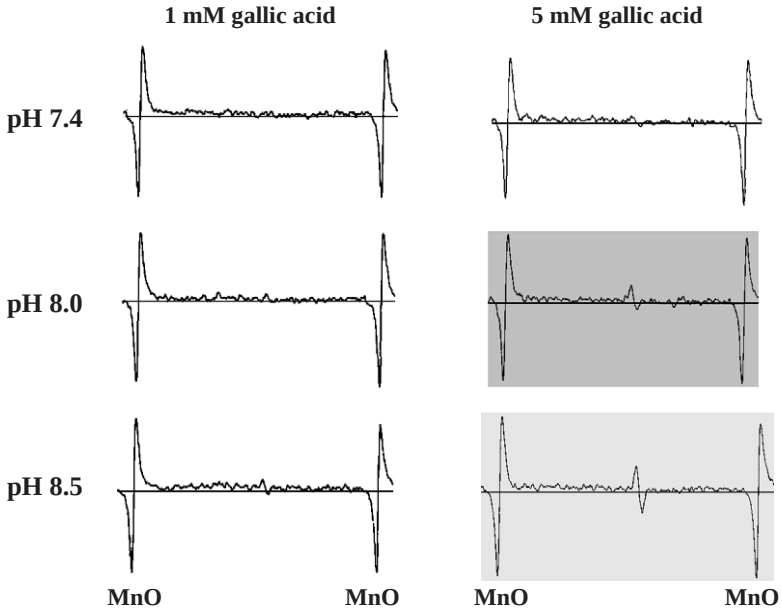

Fig. 8. Measurement of gallic acid radical by ESR spectrometer. Gallic acid (1 and 5 mM) was dissolved in 0.1 M Tris–HCl buffer (pH 7.4, 8.0, 8.5) and the electron spin resonance spectrum was measured. Instrument settings were as follows: operating power; 4 mW, center field; 336.2 mT, sweep time; 0.5 min, modulation amplitude; 0.2 mT, amplitude; 500, time constant; 0.1 s.

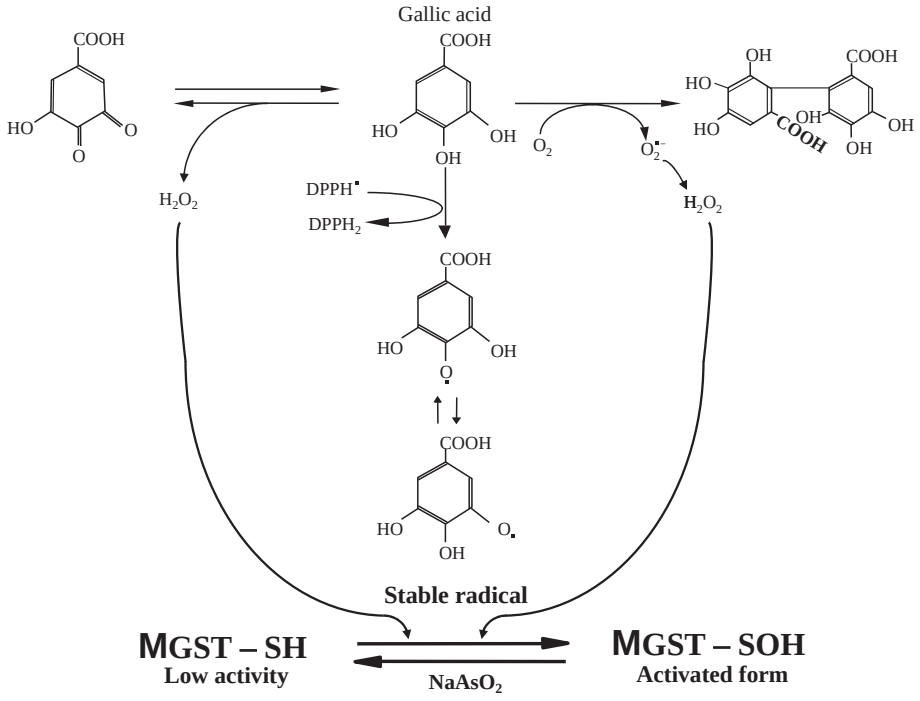

Fig. 10. Proposed mechanism of MGST1 activation by gallic acid.

sodium arsenite which can reduce sulfenic acid to thiol. As shown in Fig. 6, the MGST1 activity increased by gallic acid was decreased to near the control level by the reducing agent. It was therefore suggested that the oxidation of the SH in Cys 49 to sulfenic acid contributes at least in part to the MGST1 activation by gallic acid.

Furthermore there is another possible mechanism for gallic acid-induced MGST1 activation that phenolic metabolite including quinone binds covalently to MGST1 resulting in its activation as reported previously (Wallin and Morgenstern, 1990). We observed that gallic acid activates MGST1 in the presence of the NADPH generating system which generates ROS in the microsomes through the cytochrome P-450 system. Since the ROS generated from the reaction mixture was scavenged by gallic acid as seen by depression of CL production (Fig. 2), it was suggested that the gallic acid acts as antioxidant followed by the activation of MGST1. The finding that the MGST1 activation by gallic acid in the presence of the NADPH generating system was also depressed by SOD and catalase suggests that superoxide-related ROS may contribute to the activation of MGST1 in the conditions as well. However, these data do not rigorously exclude such a mechanism that a quinone derivative produced concomitantly by ROS generation from gallic acid activates MGST1.

We also examined whether gallic acid-induced MGST1 activation was prevented by 1,1-diphenyl-2-picrylhydrazyl (DPPH), a stable radical, which has been widely used for an evaluation of the antioxidant activity (Ratty et al., 1988). It is well known that an antioxidant (AH) scavenges DPPH radical resulting in antioxidant radical (˙A) which is quite stable. Since DPPH radical itself did not increase the MGST1 activity (Fig. 7) it was suggested that the gallic acid radical/intermediate generated from the reaction with DPPH cannot activate MGST1. These were supported by that the life span of gallic acid radical detected by ESR spectrometer was 30 min, meaning that the radical is stable to react with MGST1. Taken all together it was suggested that gallic acid generates ROS which in turn activates MGST1 rather than that gallic acid-intermediates activate MGST1. The proposed mechanism of gallic acid-induced MGST1 activation was summarized in Fig. 10.

We indicated that gallic acid derivatives such as propylgallate, laurylgallate, and pyrogallol decreased or slightly increased the MGST1 activity and ferric chloride prevented the MGST1 activation by gallic acid. Since gallic acid can act as a pro-oxidant and the free hydroxyl or carboxyl groups in gallic acid molecule contribute to its oxidation potential (Sakagami et al., 1997) it can be explained that blocking of the carboxyl group by alkylation or chelating free hydroxyl groups with ferric ion may reduce the oxidation potential of gallic acid resulting in loss of the MGST1 activation.

It was also observed that purified GSH-free MGST1 was activated dose-dependently by gallic acid (7.5-fold increase at 1 mM), confirming that gallic acid is capable of activating MGST1 directly not via microsomal membranes.

Gallic acid can enhance the anti-tumor effect of the drug in vivo (Kawada et al., 2001) or was protective against the chemical-induced hepatotoxcity (Anand et al., 1997). In consideration that MGST1 was not activated by gallic acid in the presence of GSH (data not shown), the activation may be difficult in the liver rich in GSH. However it may be activated by gallic acid in vivo in such conditions that GSH is depleted by oxidative stress. Since gallic acid is involved in diets, herbs, or various teas, the study of the effect of gallic acid on MGST1 in vivo will be encouraged.

In conclusion the gallic acid generates ROS which in turn oxidizes the SH in MGST1 resulting in the activation of the enzyme and the oxidation to sulfenic acid is involved at least in part as the activation mechanism.

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