Bull Environ Contam Toxicol (2010) 84:347–350
DOI 10.1007/s00128-010-9936-9

Evaluation of Pyrogallol-induced Cytotoxicity in Catalase-mutant *Escherichia coli* and Mutagenicity in *Salmonella typhimurium*

Yoko Takemura · Da-Hong Wang · Rani Sauriasari · Masako Horita · Ken Tsutsui · Kuniaki Sano · Noriyoshi Masuoka · Tomoko Takigawa · Jiro Takaki · Keiki Ogino

Received: 4 June 2009 / Accepted: 13 January 2010 / Published online: 26 January 2010

Abstract We evaluated pyrogallol cytotoxicity using *Escherichia coli* strains that express mammalian catalase gene derived from catalase mutant mice (Cs^b) and wild-type (Cs^a), and pyrogallol mutagenicity by Ames test. Pyrogallol was more toxic to Cs^b rather than to Cs^a ($p < 0.05$), while catalase, superoxide dismutase and ascorbic acid decrease the toxic effect. Pyrogallol also showed mutagenic effect (mutagenic index = 3.8 for 10 μmol pyrogallol/plate) while ascorbic acid (19.4% reduction, $p < 0.001$) and naringin (35.1% reduction, $p < 0.001$) played a protective role against it. Pyrogallol cytotoxicity and mutagenicity seem to be attributable, at least in part, to reactive oxygen species formation. This study also suggests that newly established catalase mutant *E. coli* is probably useful in hazard identification of oxidative chemicals.

Keywords Catalase-mutant *Escherichia coli* · Pyrogallol · Cytotoxicity · Mutagenicity

Y. Takemura · D.-H. Wang (✉) · R. Sauriasari · T. Takigawa · J. Takaki · K. Ogino
Department of Public Health, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama 700-8558, Japan
e-mail: dahong@md.okayama-u.ac.jp

M. Horita
Koka Public Health Center, 6200 Minakuchi, Minakuchi-cho, Koka 28-8511, Japan

K. Tsutsui
Department of Genomics and Proteomics, Okayama University Advances Science Research Center, 2-5-1 Shikata-cho, Okayama 700-8558, Japan

K. Sano
Department of Neuroanatomy and Neurobiology, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, 2-5-1 Shikata-cho, Okayama 700-8558, Japan

N. Masuoka
Department of Life Science, Okayama University of Science, 1-1 Ridai-cho, Okayama 700-0005, Japan

Pyrogallol (1,2,3-trihydroxybenzene) is commonly used in the hairstyling industry due to its hair straightening and permanent dyeing properties. However, pyrogallol has drawn much attention among the ingredients of hair dyes because of its autooxidation capability and for producing reactive oxygen species (ROS) (Marklund and Marklund 1974). In conducting safety evaluations studies, the European Union (EU) has evaluated pyrogallol and concluded that it could not be used as hair dye ingredient (Nohynek et al. 2004). However, there were still conflicting findings concerning its toxic and mutagenic effect and pyrogallol is still widely used in commercial hair dye formulas in many Asian countries and the United States.

In 1966, Feinstein et al. established catalase-mutant mouse strains, acatalasemic mouse ($C_3H/AnL/Cs^b$) (hereinafter called Cs^b) as animal model for evaluating ROS-induced damage. Recently, mutant catalase cDNAs from the Cs^b and its related wild-type ($C_3H/AnL/Cs^a$) (hereinafter called Cs^a) mice were successfully cloned and expressed in bacteria (Wang et al. 2001). A Gln to His substitution at amino acid position 11 of Cs^b catalase gene is responsible for the reduced activity of Cs^b catalase (Wang et al. 2001). The present study employed newly established strains of catalase normal and mutant *E. coli* and *Salmonella* mutagenicity test to examine pyrogallol-induced cytotoxicity and mutagenicity and the effects of

some enzymatic and non-enzymatic antioxidants on pyrogallol toxicity and mutagenicity.

Materials and Methods

Pyrogallol, copper superoxide dismutase (CuSOD), ascorbic acid, naringin, and quercetin were purchased from Wako Pure Chemicals (Osaka, Japan). All reagents were of the best commercially available grade. For construction of catalase mutant *E. coli* strains, we employed a *katG katE* double mutant *E. coli* UM255 as a recipient since it is completely deficient in hydroperoxidase (both HP-I and HP-II) synthesis. Catalase activity of transformant *E. coli* strain Cs^b is only half of that in Cs^a (Horita et al. 2005). Growth of inhibition test was performed by diluted the overnight cultures of tester strains in fresh LB medium and incubated at 37°C. An aliquot (30 μL) of each culture with an optical density of around 0.1 at 600 nm were added to molten top agar (3 mL) and poured on LB agar plates. Paper discs (6 mm in diameter) were placed on the solidified top agar plates, and 15 μL of pyrogallol solution were dropped onto the center of the paper discs and then all the treated plates were incubated at 37°C for 20 h. For testing the effects of antioxidants, we pretreated the LB agar plate with antioxidant solutions (100, 500, and 2,000 U for CuSOD; 25, 50, and 100 U/plate for catalase; 14 and 35 mmol/plate for ascorbic acid). We also examined pyrogallol cytotoxicity by colony-forming efficiency test as described previously (Horita et al. 2005).

Mutagenic effect of pyrogallol was evaluated by Ames test using the pre-incubation method (Yahagi et al. 1977). The Ames test employs the mutant strains of *Salmonella typhimurium*, with pre-existing mutations on the histidine operon gene that prevents them from synthesizing an essential amino acid, histidine. As a result, the bacteria cannot survive and grow without histidine. However, if a mutagenic agent is present, it can induce reverse mutation that causes the mutant bacteria to revert from being auxotrophic (*Salmonella typhmurium* His− or histidine dependent) to protrophic (*S. typhimurium* His+ or wild type that is able to synthesize histidine) (Mortelmans and Zeiger 2000). In this study, we employed *S. typhimurium* strains TA98, TA100, and TA102. Results are expressed as Mutation index (MI) that indicates the revertants of pyrogallol divided by the revertants of negative control. MI over 2 indicates mutagenicity and $1.5 < MI < 2$ indicates weak mutagenicity. We further evaluated the effects of antioxidants ascorbic acid, naringin, and quercetin by Ames test in strain TA100 with S9, in which all the experimental procedure was the same as has been mentioned except that 100 μL test sample included 50 μL pyrogallol solutions and 50 μL antioxidant solution (naringin or quercetin). Since ascorbic acid is vulnerable to heating, 700 μL of ascorbic acid solution was pretreated onto the glucose agar plate. The differences among groups in relation to Ames test were analyzed by one-way analysis of variance (one-way ANOVA) following Bonferroni tests and trend analysis with a Windows version SPSS 11.0. For assessing the differences in pyrogallol toxicity among Cs^a and Cs^b, student's *t*-test and 2-way ANOVA were used.

Results and Discussion

Growth of inhibition test showed that zone surrounding the paper disc of Cs^a was significantly smaller than that of Cs^b (Fig. 1). The viability of Cs^a and Cs^b also decreased as the concentration of pyrogallol increased ($p < 0.001$) and Cs^b was more susceptible to pyrogallol than Cs^a ($p < 0.05$) (data not shown). The addition of SOD, catalase or ascorbic acid lessened the toxic effects of pyrogallol on both strains, and the differences in diameter of inhibition zone between Cs^a and Cs^b became smaller (Fig. 1). In particular, the addition of 100 U/plate catalase abolished the toxicity caused by pyrogallol. It is known that catalase is supposed to play a critical role in detoxification of H_2O_2 in *E. coli*, since no glutathione peroxidase has been identified in *E. coli* (Smith and Schrift 1979). The present results further provide evidence that H_2O_2 is one of mediators of pyrogallol-induced cytotoxicity. Pretreatment with SOD as well as ascorbic acid also inhibited pyrogallol toxicity dose-dependently in both strains, suggesting a possibility of $O_2^{\bullet -}$ formation in the process of pyrogallol toxicity. SOD is an enzyme that can catalyze the conversion of $O_2^{\bullet -}$ to H_2O_2, while ascorbic acid is known to have

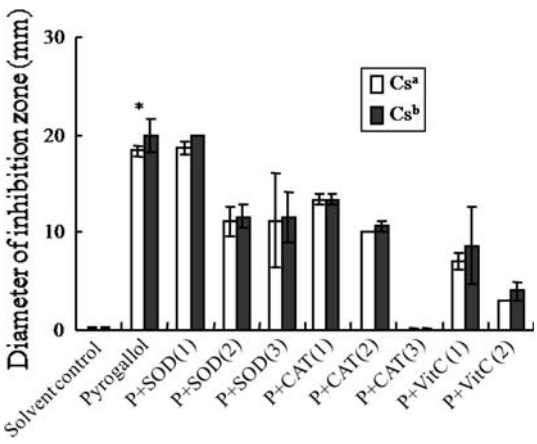

Fig. 1 Effect of antioxidants on pyrogallol-induced cytotoxicity. Each *bar* represents mean ± SD ($N > 3$) of diameters of inhibiting zone. CuSOD (1): 100 U/plate, (2): 500 U/plate, (3): 2,000 U/plate; CAT (1): Catalase 25 U/plate, (2): 50 U/plate, (3): 100 U/plate; Vit C (1): Ascorbic acid 14 mmol/plate, (2): 35 mmol/plate. *$p < 0.05$

the ability to act as a scavenger of $O_2^{\bullet -}$ and $OH^{\bullet}$ (Grey and Adlercreutz 2003).

Our data also showed that pyrogallol was mutagenic to TA100 in the presence of metabolic activation, whereas in the absence of metabolic activation, pyrogallol was highly mutagenic to TA98 and TA100, and weakly mutagenic to TA102 (Table 1).

The mutagenicity of pyrogallol observed in the present study is decreased in the presence of S9 mix as metabolic activation inducer, which is consistent with the report by Do Céu Silva et al. (2003). The disagreements on mutagenic effect of pyrogallol between the results of ours and that of some studies in the literature (Lin and Lee 1992; Glatt et al. 1989) were possibly due to the difference in the treatment and concentrations used. Furthermore, ascorbic acid and naringin suppressed pyrogallol mutagenicity (Table 2) and the protective effect of ascorbic acid was more marked than that of naringin.

Kanno et al. (2003) demonstrated that naringin was protective against H_2O_2-induced cytotoxicity and apoptosis in P388 cells previously, and our study is the first one that further proved the protective role of naringin against pyrogallol. In contrary, quercetin highly augmented the mutagenicity of pyrogallol. Although quercetin in low concentration inhibited oxidative damage by scavenging H_2O_2, $O_2^{\bullet -}$, and $OH^{\bullet}$ (Horváthová et al. 2005), it also has been reported having pro-oxidant effect through the

Table 2 Effects of ascorbic acid, naringin and quercetin on pyrogallol-induced mutagenicity in *Salmonella typhimurium* TA100 with metabolic activator S9

Concentration (per plate)	% of induction[#] (%)
Ascorbic acid	
7 mmol	61.8 ± 7.3**
14 mmol	57.7 ± 5.1**
28 mmol	68.9 ± 3.7**
56 mmol	80.6 ± 6.9**
Naringin	
2.5 nmol	75.1 ± 3.8*
5 nmol	76.1 ± 6.7
50 nmol	69.3 ± 13.0*
250 nmol	64.9 ± 9.9**
Quercetin	
50 nmol	116.7 ± 13.5
125 nmol	107.0 ± 13.0
250 nmol	155.3 ± 6.5
500 nmol	162.9 ± 5.9*
1 μmol	184.3 ± 41.3*

Data are expressed as mean ± SD (N > 3)

Each plate contained 3.5 μmol of pyrogallol

[#] Percent of induction indicates the revertants/plate containing pyrogallol and antioxidants divided by revertants/plate containing pyrogallol only

* $p < 0.05$, ** $p < 0.01$ compared with pyrogallol treatment alone

Table 1 Mutagenicity of pyrogallol

Concentration (per plate)	+S9						−S9					
	TA98[a]		TA100[a]		TA102[b]		TA98[c]		TA100[c]		TA102[b]	
	$His^{+\#}$	$MI^{\#\#}$	$His^{+\#}$	$MI^{\#\#}$	$His^{+\#}$	$MI^{\#\#}$	$His^{+\#}$	$MI^{\#\#}$	$His^{+\#}$	$MI^{\#\#}$	$His^{+\#}$	$MI^{\#\#}$
Pyrogallol												
0	20 ± 8	–	59 ± 11	–	487 ± 43	–	32 ± 3	–	81 ± 6	–	351 ± 23	–
1 nmol	28 ± 5	1.4	69 ± 2	1.1	548 ± 62	1.1					464 ± 31	1.3
10 nmol	22 ± 5	1.1	77 ± 5	1.3	542 ± 47	1.1					527 ± 39	1.5
100 nmol	26 ± 1	1.3	87 ± 6	1.4	582 ± 96	1.2					547 ± 116	1.6
1 μmol	27 ± 4	1.4	157 ± 20	2.6	456 ± 80	0.9					550 ± 11	1.6
2 μmol							30 ± 1	0.9	166 ± 20	2.1		
4 μmol							51 ± 3	1.2	310 ± 46	3.8		
6 μmol							357 ± 85	11.2	Toxic	0		
8 μmol							Toxic	0	Toxic	0		
10 μmol	11 ± 9	0.6	227 ± 41	3.8	177 ± 23	0.4					Toxic	0
Mutagenicity	–		+		–		+		+		±	

Trend analysis was performed excluding the plates showing biocide effect. TA98 (+S9): $\eta^2 = 0.425$, $p < 0.01$, TA100: $\eta^2 = 0.734$, $p < 0.001$, TA102: $\eta^2 = 0.813$, $p < 0.001$. TA98(−S9): $\eta^2 = 0.62$, $p < 0.001$, TA100: $\eta^2 = 0.909$, $p < 0.001$, TA102: $\eta^2 = 0.217$, $p < 0.05$

[#] His^+ indicates His^+ revertants per plate. All data are expressed as mean ± SD (N = 3). Assays for each concentration of pyrogallol were performed using two or three plates for each experiment

[##] The mutagenicity of pyrogallol was obtained by counting the number of His^+ revertants/plate. MI indicates the revertants of pyrogallol divided by the revertants of negative control. MI > 2 indicates the mutagenicity (+), and 1.5 < MI < 2 indicates the weak mutagenicity (±)

[a] 100 nmol/plate 2-NF for positive control; [b] 25 nmol/plate 4NQO for positive control; [c] 50 nmol/plate 2-NF for positive control

generation of $O_2^{\bullet -}$, H_2O_2 and $OH^{\bullet}$ in a Fenton reaction system (Kessler et al. 2003). Quercetin also has a mutagenic effect that can be enhanced in the presence of S9 (Brown 1980). This results revealed that naringin and quercetin, even in the same group of polyphenolic flavonoids, exhibited different effects, because the antioxidant activities of flavonoids have been suggested to be structure-related (Wang et al. 2006).

The present results suggest that pyrogallol-induced cytotoxicity and mutagenicity seems to be attributable to the formation of ROS. Both enzymatic (catalase, SOD) and non-enzymatic (ascorbic acid, naringin) antioxidants played a protective role against the pyrogallol-induced cytotoxicity and mutagenicity. This study also suggests that newly established catalase mutant *E. coli* is probably useful in hazard identification of oxidative chemicals.

Acknowledgments We would like to express our gratitude to Dr. P. C. Loewen for kindly supplying bacterial strain UM255. This work was supported by Grant-in-Aid for Science Research from the Ministry of Education, Science and Culture of Japan.

References

Brown JP (1980) A review of the genetic effects of naturally occurring flavonoids, anthraquinones and related compounds. Mutat Res 75:243–277

Do Céu Silva M, Gaspar J, Silva ID, Leão D, Rueff J (2003) Induction of chromosomal aberrations by phenolic compounds: possible role of reactive oxygen species. Mutat Res 540:29–42

Feinstein RN, Howard JB, Braun JT, Seaholm JE (1966) Acatalasemic and hypocatalasemic mouse mutants. Genetics 53:923–933

Glatt H, Padykula R, Berchtold GA, Ludewig G, Platt KL, Klein J, Oesch F (1989) Multiple activation pathways of benzene leading to products with varying genotoxic characteristics. Environ Health Perspect 82:81–89

Grey CE, Adlercreutz P (2003) Ability of antioxidants to prevent oxidative mutations in *Salmonella typhimurium* TA102. Mutat Res 527:27–36

Horita M, Wang DH, Tsutsui K, Sano K, Masuoka N, Kira S (2005) Involvement of oxidative stress in hydroquinone- induced cytotoxicity in catalase-deficient *Escherichia coli* mutants. Free Radic Res 39:1035–1041

Horváthová K, Chalupa I, Šebová L, Tóthová D, Vachálková A (2005) Protective effect of quercetin and luteolin in human melanoma HMB-2 cells. Mutat Res 565:105–112

Kanno S, Shouji A, Asou K, Ishikawa M (2003) Effects of naringin on hydrogen peroxide-induced cytotoxicity and apoptosis in P388 cells. J Pharmacol Sci 92:166–170

Kessler M, Ubeaud G, Jung L (2003) Anti- and pro-oxidant activity of rutin and quercetin derivatives. J Pharm Pharmacol 55:131–142

Lin JK, Lee SF (1992) Enhancement of the mutagenicity of polyphenols by chlorination and nitrosation in *Salmonella typhimurium*. Mutat Res 269:217–224

Marklund S, Marklund G (1974) Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. European J Biochem 47:469–474

Mortelmans K, Zeiger E (2000) The ames *salmonella*/microsome mutagenicity assay. Mutat Res 455:29–60

Nohynek GJ, Fautz R, Benech-Kieffer F, Toutain H (2004) Toxicity and human health risk of hair dyes. Food Chem Toxicol 42:517–543

Smith J, Schrift A (1979) Phylogenic distribution of glutathione peroxidase. Comp Biochem Physiol 63:39–44

Wang DH, Tsutsui K, Sano K, Masuoka N, Kira S (2001) cDNA cloning and expression of mutant catalase from the hypocatalasemic mouse: comparison with the acatalasemic mutant. Biochim Biophys Acta 1522:217–220

Wang L, Tu YC, Lian TW, Hung JT, Yen JH, Wu MJ (2006) Distinctive antioxidant and antiinflammatory effects of flavonols. J Agric Food Chem 54:9798–9804

Yahagi T, Nagao M, Seino Y, Matsushima T, Sugimura T (1977) Mutagenicities of N-nitrosamines on *Salmonella*. Mutat Res 48:121–129