The Journal of Toxicological Sciences (J. Toxicol. Sci.)
Vol.33, No.2, 197-207, 2008

Original Article

Modifying effect of *Siraitia grosvenori* extract on piperonyl butoxide-promoted hepatocarcinogenesis in rats

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(Received December 3, 2007; Accepted January 7, 2008)

ABSTRACT — To examine the possible modifying effect of the extract of *Siraitia grosvenori* (SGE), a naturally occurring antioxidative agent, on piperonyl butoxide (PBO)-promoted hepatocarcinogenesis, male F344 rats were administered a single intraperitoneal injection of *N*-diethylnitrosamine (DEN) as an initiator followed by administration of a diet containing 2% PBO for 7 weeks with or without SGE (1,000 ppm) in the drinking water. To enhance cellular proliferation, all animals underwent two-thirds partial hepatectomy 1 week after the commencement of PBO administration. Pretreatment with SGE was also applied to the PBO + SGE group for 2 weeks prior to DEN initiation. Liver immunohistochemistry revealed that although the PBO-mediated increase in the number of glutathione *S*-transferase placental form (GST-P)-positive foci and proliferating cell nuclear antigen-positive cells remained unaltered with SGE coadministration, the area of the GST-P-positive foci was increased. On the contrary, real-time RT-PCR showed that coadministration of SGE increased hepatic GST and glutathione peroxidase (GSH-Px) antioxidant activities and mRNA expression levels of the phase II enzymes that are known to be transcriptionally up-regulated through the Nrf 2-Keap1-antioxidant responsive element (ARE) as well as the phase III enzymes. Furthermore, measurement of thiobarbituric acid-reactive substances showed a decrease in lipid peroxidation by SGE coadministration. The results suggest that SGE may exert hepatic antioxidant activity by up-regulating the genes under the control of the Nrf 2-Keap1-ARE transcriptional machinery; however, this activity was neither effective nor sufficient for suppression of PBO-promoted early hepatocarcinogenesis.

Key words: Piperonyl butoxide, Oxidative stress, *Siraitia grosvenori*, Antioxidant, Rat

INTRODUCTION

Piperonyl butoxide, α-[2-(2-butoxyethoxy)ethoxy]-4,5-methylenedioxy-2-propyltoluene (PBO), a pesticide synergist, is widely used along with pyrethroids as a grain protector and domestic insecticide. PBO has been reported to act as a hepatocarcinogen in F344 rats fed on a 1.2% or 2.4% PBO-containing diet for 2 years (Takahashi *et al.*, 1994). However, PBO has been shown to be negative in bacterial mutation assays, hypoxanthine-guanine phosphoribosyltransferase (HGPRT) assay, chromosomal aberrations test performed using Chinese hamster ovary cells, and *in vitro* unscheduled DNA synthesis test (Beamand *et al.*, 1996; Butler *et al.*, 1996). In a two-stage rat hepatocarcinogenesis model, PBO has been reported to act as a liver tumor promoter in F344 rats (Okamiya *et al.*, 1998). PBO acts as a mild cytochrome P450 (CYP) 1A1 inducer by inefficient activation of the aryl hydrocarbon receptor (AhR). CYP1A1 is also known to play an important role in the chemical activation of xenobiotics to carcinogenic derivatives. Our previous study demonstrated that PBO has the potential to generate reactive oxygen species (ROS) by induction of CYP1A1 and suggested the possibility that DNA damage resulting from oxidative stresses due to ROS generation is involved in the mechanism of hepatocarcinogenesis by PBO in rats and mice

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(Muguruma *et al.*, 2007).

Siraitia grosvenori, a traditional Chinese fruit, belongs to the cucurbitaceous species and has been used as a folk medicine for sore throat, cough, and minor stomach and intestinal problems. It contains several sweet triterpene glycosides. Recently, the extract of *Siraitia grosvenori* (SGE) has been shown to exert a variety of physiological functions. For example, SGE reduces the atherogenic potential of low-density lipoprotein (LDL) by dose-dependent inhibition of copper-mediated oxidation and human umbilical vein endothelial cell-mediated LDL oxidation (Takeo *et al.*, 2001). SGE has also been shown to exert free radical scavenging activity (Shi *et al.*, 1996) and to possess antiallergenic potential in a mouse model of histamine-induced nasal rubbing and compound 48/80-induced skin scratching (Hossen *et al.*, 2005). Treatment of diabetic mice with SGE also prevented the development of diabetic nephropathy owing to its antioxidative action (Song *et al.*, 2006). In addition, cucurbitane triterpene glycosides in SGE, namely, mogroside V and 11-oxo-mogroside V, have been shown to inhibit the tumor-promoting activity of 12-*O*-tetradecanoylphorbol-13-acetate in a two-stage mouse skin carcinogenesis model; they have also been shown to inhibit the tumor-initiating activity of peroxynitrite (Takasaki *et al.*, 2003).

In view of the antioxidative properties of SGE, we, in the present study, investigated (1) the modifying effect of SGE on PBO-promoted hepatocarcinogenesis in rats and (2) the molecular events related to its antioxidative responses in a two-stage rat hepatocarcinogenesis model.

MATERIALS AND METHODS

Chemical, animals, and treatment

PBO (CAS 51-03-6; technical grade; purity, > 90%) was purchased from ACROS Organics (Morris Plains, NJ, USA). SGE was kindly supplied by the biochemical laboratory Saraya Co., Ltd. (Osaka, Japan). A total of 36 male F344/N Slc rats aged 4 weeks and weighing 53.75 ± 6.19 g were purchased from Japan SLC Inc. (Shizuoka, Japan). They were assigned to the control, PBO-treated (PBO) or PBO plus SGE-treated (PBO + SGE) groups. The dietary concentration of SGE was selected as 1,000 ppm based on the result of the preliminary study in which rats were fed with a diet containing 2% PBO and water containing SGE at 40 ppm, 200 ppm or 1,000 ppm for 4 weeks (unpublished observations). In the preliminary study, a dose-dependent increased expression of phase II drug-metabolizing enzyme genes was observed in real-time RT-PCR analyses. Therefore the dose of SGE was determined to be 1,000 ppm. Four rats were housed in each stainless steel cage under the conventional conditions (12-hr light/dark cycle; relative humidity, 55% ± 5%; temperature, 22 ± 2°C, i.e., room temperature), and rats of the control and PBO groups or PBO + SGE group allowed *ad libitum* access for 2 weeks to tap water containing 0 or 1,000 ppm SGE. All rats were provided a commercial powdered-basal diet (MF, Oriental Yeast Industries Co., Ltd., Tokyo, Japan) for 2 weeks. In this experiment, we employed a two-stage rat liver carcinogenesis model in a medium-term rat liver bioassay (Fig. 1; Shirai, 1997). All animals were administered a single intraperitoneal injection of *N*-diethylnitrosamine (200 mg/kg body weight) dissolved in saline in order to initiate hepatocarcinogenesis. Two weeks after injection, the animals of the PBO and PBO + SGE groups were administered a powdered diet containing 2% PBO for 7 weeks. This treatment period is generally 6 weeks, but the treatment period was changed to 7 weeks because of the man-power reason for the conduct of various examinations at the final necropsy. In addition, animals of the PBO + SGE group were subjected to concurrent administration of water containing 1,000 ppm SGE for 7 weeks. The PBO dose was determined based on our previous study, which was also conducted on a two-stage rat hepatocarcinogenesis model (Muguruma *et al.*, 2007). In order to enhance hepatocellular proliferation, all rats were subjected to two-thirds partial hepatectomy at 1 week after PBO feeding. Seven rats died postoperatively within 1 week and 29 rats survived (8 rats in the control group, 10 in the PBO group, and 11 rats the PBO + SGE group). Body weight and food consumption were measured once a week. The experiment was performed in accordance with the guidelines for animal experimentation of the Faculty of Agriculture, Tokyo University of Agriculture and Technology. At the end of the treatment period, 3 animals from each group were used for the measurement of microsomal ROS production. Other animals were killed by exsanguination from the abdominal aorta under ether anesthesia and necropsied. Their livers were excised, weighed and either fixed in neutral buffered formalin or in Methacarn (methanol:chloroform:acetic acid = 6:3:1) for histopathological examination or cut into small pieces, frozen in RNA *later* (QIAGEN, Hilden, Germany), and stored at -80°C until analysis.

Histopathology and morphometry

The livers of all animals from the control, PBO and PBO + SGE groups were examined histopathologically. The livers that were fixed in Methacarn were embedded in paraffin wax, sectioned and stained with hematoxylin

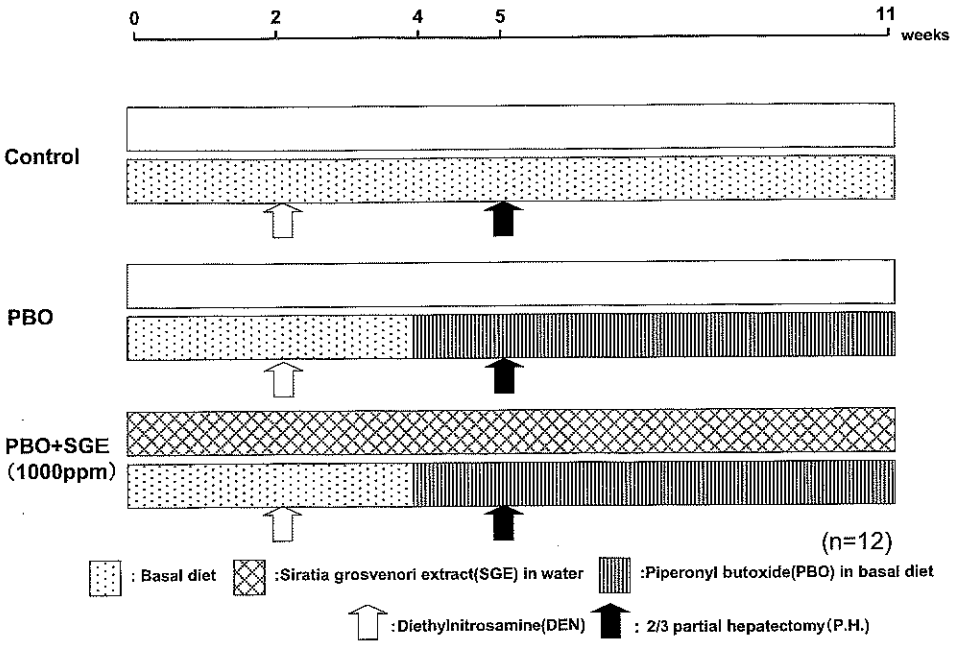

Fig. 1. Experimental design.

and eosin. Schmorl staining was additionally performed for the determination of lipofuscin deposition in the livers of the animals.

Immunohistochemistry

The sections fixed in Methacarn were used for immunohistochemical staining for glutathione *S*-transferase placental form (GST-P) and proliferating cell nuclear antigen (PCNA) by the avidin-biotin complex method. Immunostaining for GST-P was performed according to a previously described method (Muguruma *et al.*, 2007). Since one rat of the control group had severe bile duct proliferation due to the surgical error of partial hepatectomy, this animal was excluded from the immunohistochemical evaluation. The GST-P-positive liver cell foci in each group (4 rats in the control group, 7 in the PBO group and 8 in the PBO + SGE group) were determined by quantitative analysis using a computer-assisted microscope (EX51; OLYMPUS Corp., Tokyo, Japan). The numbers and areas of the foci with cross-sectional areas > 0.01 mm^2 and the total area of the liver sections were measured using a computer-assisted image analyzer (NIH Image, a free PC version of Image, called as Scion Image for Windows available from Scion Corporation) to obtain the respective values in terms of per cm^2 of the liver section.

For immunostaining for PCNA, deparaffinized liver sections were denatured by immersing in 2 N HCL for 30 min in order to hydrolyze DNA. The sections were then rinsed in 2 changes of distilled water and phosphate-buffered saline (pH 7.4). Immunostaining was performed using a mouse anti-PCNA antibody (Clone PC10; 1:300; DAKO, Glostrup, Denmark). The number of PCNA-positive cells among approximately 2,000 hepatocytes was then counted.

Measurement of microsomal ROS production in the liver

The liver microsomes were obtained from 3 rats of each group. ROS production was measured as described previously (Muguruma *et al.*, 2006). To examine ROS generation in relation to metabolic activation by CYPs, the pooled sample from the PBO group was treated with SKF525A, a non-specific P450 inhibitor of mixed-function oxidases. The formation of ROS was detected as a fluorescent signal and was measured by a microplate reader (excitation, 485 nm; emission, 528 nm).

Determination of TBARS content

Lipid peroxidation in the livers was assessed by quantitation of the generated thiobarbituric acid-reactive substances (TBARS; Wasoxicz *et al.*, 1993) in each group (4 rats in the control group, 6 in the PBO group and 6 in the PBO + SGE group). Briefly, 100 μl of the liver homogenate in 1.15% KCl, 100 μl of 8.1% sodium dodecyl sul-

phate, 750 µl of 20% acetic acid (pH 3.5), 25 µl of 0.8% butylated hydroxytoluene, 750 µl of 0.8% thiobarbituric acid and 350 µl of distilled water were mixed in that order, heated at 95°C for 60 min, and then cooled. The reaction mixture was centrifuged at 3,000 rpm for 10 min after adding 0.5 ml of distilled water and 2.5 ml *n*-butanol and pyridine (15:1 v/v). The absorbance of the resulting solution was determined spectrophotometrically at 532 nm using the Synergy HT Multi-Detection Microplate Reader (BioTek, VT, USA).

Determination of GST and GSH-Px enzyme activities

The mixture used for measuring glutathione *S*-transferase (GST) activity contained 1 mM glutathione, 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) in ethanol, 0.025 ml of cytosolic sample (containing 20 µg of protein) and 0.1 M Na^+/Ka^+ phosphate buffer (pH 6.5). The product obtained from the reaction between the thiol group of glutathione and the electrophilic group of CDNB was read spectrophotometrically at 340 nm ($\varepsilon = 9.6\ mM^{-1}cm^{-1}$) (Habig *et al.*, 1974). CDNB is a wide range of GST substrate, especially GST-*m1* and GST-*m3* (Jensson *et al.*, 1986, Mannervik *et al.*, 1985). The mixture used for measuring glutathione peroxidase (GSH-Px) activity contained 200 U/ml glutathione reductase, 20 mM glutathione, 10 mM EDTA, 10 mM NaN_3, 5 mM NADPH, 5 mM H_2O_2 and 0.1 M Na^+/Ka^+ phosphate buffer (pH 7.0). The decrease in absorbance of NADPH was measured at 340 nm for 4 min ($\varepsilon = 6.22 \times 10^3\ M^{-1}cm^{-1}$) (Flohe *et al.*, 1984).

DNA microarray analysis

As a screening analysis to examine fluctuations of variable genes attributable to coadministration of PBO and SGE, the livers from 1 PBO-treated and 1 PBO + SGE-treated rat were each analyzed by Oligo GEArray Rat Toxicology and Drug Resistance Microarray (ORN-401; SuperArray Bioscience Corp., Frederick, MD). Total RNA was extracted using TRIzol (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. By using the TrueLabeling-AMP kit (SuperArray Bioscience Corp.), cRNA was synthesized from 3 µg of total RNA by conversion of the total RNA to a biotin-labeled cRNA probe through the cDNA synthesis step. After purification of the biotin-labeled cRNA probes by using the ArrayGrade™ cRNA Clean-up Kit (SuperArray Bioscience Corp.), the array membranes were hybridized overnight with the biotin-labeled probes at 60°C. The membranes were then washed twice with 2 × saline-sodium citrate buffer (SSC)/1% sodium dodecyl sulfate (SDS) followed by washing twice with 0.1 × SSC/1% SDS at 60°C for 15 min each. Chemiluminescent detection steps were performed by subsequent incubation of the membranes with alkaline phosphatase-conjugated streptavidin and CDP-Star substrate followed by exposure to Hyperfilm™ ECL X-ray film (Amersham Biosciences UK Ltd., Buckinghamshire, UK). The image data obtained from GEArray were analyzed using the GEArray Expression Analysis Suite software (http://www.geasuite.superarray.com/index.jsp) after correcting for background noise by subtraction of the minimum value and normalizing to the value of 2 housekeeping genes (*Ppia and GAPDH*). For each spot, the ratio of intensities between the PBO and PBO + SGE groups was analyzed. Gene expressions in the PBO + SGE group were considered to be significant if their fold change values were > 1.5-fold, as compared to the PBO group.

Real-time RT-PCR

Real-time RT-PCR analysis was performed using liver tissue from each group (4 animals in the control group, 5 in the PBO group and 5 in the PBO + SGE group). Since one rat of the control group had severe bile duct proliferation due to the surgical error of partial hepatectomy, this animal was excluded from the real-time RT-PCR. Based on the results of the present cDNA microarray analyses and our previous study on PBO-induced hepatocarcinogenesis (Muguruma *et al.*, 2007), genes related to oxidative stress were selected for quantitative real-time RT-PCR analyses. cDNA was synthesized from 2 µg of RNA in the presence of dithiothreitol (DTT), dNTPs, random primers, RNaseOUT and SuperScript™ III Reverse Transcriptase (Invitrogen Corp.) in 20 µl total reaction mixture. Real-time RT-PCR was performed using the SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and the ABI PRISM 7000 Sequence Detection System (Applied Biosystems) to validate the microarray results, according to the SYBR® Green PCR Master Mix protocol. The PCR primers shown in Table 1 were designed using the Primer Express software (Applied Biosystems). The amount of transcripts of the target genes normalized to an endogenous reference gene, *Myo1b*, and relative to a control was determined by the $^{2\Delta}CT$ method (Livak and Schmittgen, 2001).

Statistical analysis

Statistical analyses were performed using a statistical software (StatLight; Yukms Co., Ltd., Japan), and all results have been presented as the mean ± SD. The significance level of the difference between the control and the PBO or PBO + SGE groups, and that between the PBO

Table 1. Sequence of primers used for real-time RT-PCR analysis.

Accession no.	Symbol	Forward primer	Reverse primer
NM_080681	Abcc3	TCCCACTTCTCGGAGACAGTAAC	CTTAGCATCACTGAGGACCTTGAA
NM_013215	Afar	CCGCTTCTTTGGGAATCCAT	GGCGATGCCATTGAAGTGT
AA800587	Gpx2	AC CG ATC CC AAG CTC ATC ATC AT	TCTCAAAGTTCCAGGACACATCTG
X78847	Gst-yc2	AAGCTGAGCAGGGCTGATGT	ACAATGCCTGGGTCCATCTC
NM_017013	Gst-a2	CAACTACATCGCCACCAAATATG	GCCTTCGGTGTACATGTCAATC
NM_017014	Gst-m1	GAACGTTCGCGGACTTACTCA	ACGTATCTCTTCTCCTCATAGTTTGAATCT
NM_031154	Gst-m3	GCCATCCTGCGCTATCTTG	CACGAATCCTCTCCTCTTCTGTCT
M13506	UDPGTR-2	CTGAAGCAGAGCCCTGAGAGA	GGGAAGGCACTGGCATGA
NM_057105	Ugt1a6	TGGCTACCCCAAAACGATCT	ATACCATGGGAACCGGAGTGT
NM_017000	Nqo1	TCCGCCCCCAACTTCTG	TCTGCGTGGGCCAATACA
NM_053906	Gsr	GCCCACGGTTCCTCATGA	GAAAGAACCCATCACTGGTTATCC
NM_017353	Slc7a5	CCTACGGAGGATGGAACTATCTGA	TGGGCAAGGAGATGATGATG
NM_012541	Cyp1a1	This gene primer set was obtained from Superarray Bioscience	
NM_031762	Cdkn1b	TTCCGCCTGCAGAAACCTCTT	TCTCGGCAGTGCTTCTCCAAGT
NM_024127	Gadd45a	CACCATAACTGTCGGCGTGTA	GGCACAGGACCACGTTGTC
NM_022246	Rad50	TGGCCCCTGGCAGTGA	AACTTCGCACGCCCAGAGT
NM_012600	ME1	CGACCAGCAAAGCTGAGTGTT	CTGCCGCTGGCAAAGATC
NM_013149	AhR	CATCCTGGAAATTCGAACCAA	GCATCACAGCCAATAGGTGTGA
NM_053986	Myo1b	GCAGGAGAAAGTTTCAACCACAT	AACCGGCTGTAGAGGTTTTTAGC

and PBO + SGE groups were determined by Student's *t*-test when the intergroup variance was proved to be homogeneous using the test for equal variance. If a significant difference was observed in the variance, Welch's *t*-test was performed. A P value of less than 0.05 was considered to be statistically significant.

RESULTS

Body and liver weights

The body weight gain in the PBO-treated rats was significantly inhibited compared with that in the control group, as shown in our earlier study (data not shown; Muguruma *et al.*, 2007). As compared to the control group, the final body weight was significantly decreased in both PBO and PBO + SGE groups. However, the values did not differ between the PBO-treated groups (Table 2). Both absolute and relative liver weights were significantly increased in both the PBO-treated groups as compared to the control group (Table 2). However, none of the values differed between the PBO and PBO + SGE groups.

Histopathology

Centrilobular hepatocytic hypertrophy was evident on microscopic examination of the livers of the PBO and PBO + SGE groups (data not shown). In these groups, the hepatocytes were enlarged due to an increase in the cytoplasmic area. Schmorl staining revealed an increase in the deposition of lipofuscin, one of the markers of oxidative stress response, in the PBO and PBO + SGE groups (Fig. 2a-c). However, there was no marked difference in the severity between these two groups.

Immunohistochemical analysis

The data for immunohistochemical analysis of the

Table 2. Body and liver weights of rats given PBO and/or SGE after DEN initiation.

	No. of rats examined	Body weight (g)	Liver weight	
			Absolute (g)	Relative (%)
Control	4	273.63 ± 16.8	6.86 ± 0.68	2.5 ± 0.21
PBO	7	209.82 ± 12.8**	11.74 ± 0.77**	5.6 ± 0.12**
PBO+SGE	8	205.55 ± 8.69**	11.66 ± 0.64**	5.67 ± 0.13**

Results are mean ± S.D. for indicated number of animals for each group.
** $P < 0.01$ vs. Control.

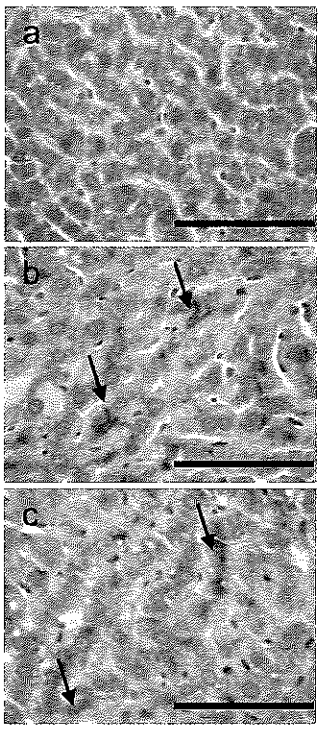

Fig. 2. Representative light microscopic views of Schrmol staining of the liver in each group (a: control, b: PBO, c: PBO + SGE). Lipofuscin deposition (arrows) in the liver of the animals of the PBO and PBO + SGE groups is more prominent as compared to the control group. Bar = 200 μm.

GST-P-positive liver cell foci and PCNA-positive cells are shown in Table 3. The number and area of GST-P-positive foci were significantly increased in both PBO and PBO + SGE groups as compared to the control group. When the values of both PBO-treated groups were compared, a significant increase was observed in the area but not in the number of the GST-P-positive foci in the PBO + SGE group. The PCNA-positive cell ratio was significantly increased in both the PBO-treated groups as compared to that in the control group; however, the values did not differ between the PBO and PBO + SGE groups.

Generation of ROS

Measurement of ROS production in the cell-free system of isolated liver microsomes obtained from the PBO and PBO + SGE groups revealed a significant increase in ROS production as compared to the control group (Fig. 3). However, ROS production did not differ significantly between the PBO and PBO + SGE groups. Addition of SKF525A to the pooled sample from the PBO group showed inhibition of ROS production.

Measurement of TBARS

Hepatic TBARS levels, measured using malondialdehyde-a metabolite of oxidized lipids-as a standard, reflect the extent of lipid peroxidation. TBARS values were significantly increased in both PBO and PBO + SGE groups as compared to that in the control group (Table 4). However, the TBARS value in the PBO + SGE group was significantly lower than that in the PBO group.

GST and GSH-Px activity

Hepatic GST activity was significantly increased in the PBO and PBO + SGE groups (Table 4), and was significantly higher in the latter as compared to the former. Hepatic GSH-Px activity in the PBO and control groups was similar. However, that in the PBO + SGE group was significantly higher than the control and PBO groups (Table 4).

cDNA microarray and real-time RT-PCR analysis

As compared to the PBO group, in the PBO + SGE group, microarray analysis of the liver showed a > 1.5-fold increase in the mRNA levels of cyclin-dependent kinase inhibitor 1B (*Cdkn1b*), growth arrest and DNA-damage-

Table 3. Quantitative data for GST-P-positive foci and PCNA-positive cells in livers of rats given PBO and/or SGE after DEN-initiation.

	No. of rats examined	GST-P-positive foci (> 0.01mm²)		PCNA-positive cells
		Numbers (No./cm²)	Areas (mm²/cm²)	Number (No./2000 cells)
Control	4	29.99 ± 4.87	0.57 ± 0.13	51.6 + 6.5
PBO	7	70.48 ± 23.49**	1.59 ± 0.39**	112.3 + 34*
PBO+SGE	8	79.6 ± 17.97**	2.75 ± 1.22**, #	107.8 + 30.9*

Results are mean ± S.D. for indicated number of animals for each group.
*,** $P < 0.05$ or $P < 0.01$vs. Control, respectively.
# $P < 0.05$ vs. PBO

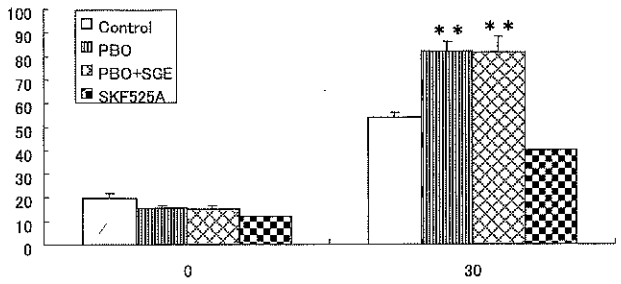

Fig. 3. Effects of PBO on hepatic microsomal ROS production. The column represents the mean ± SD. Hepatic microsomes isolated from the control, PBO and PBO + SGE groups were measured in a cell-free system (n = 3 in each group). The pooled sample from the PBO group was used for examination of the effect of SKF525A, a nonspecific P450 inhibitor of mixed-function oxidases. **$P < 0.01$ vs. the control group.

inducible 45 alpha (*Gadd45a*), rat homolog of Rad50 (*Rad50*), epoxide hydrolase 1, microsomal, (*Ephx1*), NAD(P)H dehydrogenase quinone 1 (*Nqo1*), glutathione reductase (*Gsr*), glutathione peroxidase 2 (*Gpx2*), and the GST isoforms pi2, mu3, and theta 1 (*Gstp2, Gstm3,* and *Gstt1*) (Table 5). To validate the mRNA expression data obtained from the microarray analysis, real-time RT-PCR analysis was performed. In addition, several xenobiotic-metabolizing genes associated with oxidative stress responses and the genes that were found to be up-regulated in our previous study on PBO (Muguruma *et al.*, 2007) were chosen, and consequently, 16 genes in all were selected for real-time RT-PCR analysis. Gene expression levels obtained by real-time RT-PCR were mostly parallel to those obtained by microarray analysis. Results of these analyses are summarized in Table 6. As reported in our previous study, mRNA levels of the phase II enzyme genes, such as *Nqo1, Gsr,* UDP-glucuronosyltransferase phenobarbital-inducible form (*Udpgtr-2*), aflatoxin B1 aldehyde reductase (*Afar*) and *Gpx2*, were significantly increased in the livers of the rats from both the PBO-treated groups, as compared to the control group. These genes were further up-regulated and their mRNA levels, significantly increased in the PBO + SGE group, as compared to the PBO group. Similarly, the mRNA expression of ATP-binding-related genes, such as *Abcc3* and oxidoreductase activity-related genes *(Me1)*, which were significantly up-regulated in the PBO group were further increased in the PBO + SGE group. On the other hand, a phase I enzyme gene, i.e., *Cyp1a1*, showed significant up-regulation in the PBO and PBO + SGE groups compared to the control group, as in our previous study (Muguruma *et al.*, 2007). However, no significant difference was observed in its expression between the PBO and PBO + SGE groups. In addition, the transcription level of *AhR*, which is related to the induction of *Cyp1a1* expression, was also significantly increased in the PBO and PBO + SGE groups, as compared to the control group. The mRNA levels of Nrf 2-dependent genes, such as *Gst* genes (*Gsta2*, *Gstm1, Gstm3,* and *Gstyc2)*, were also increased in the PBO group, as compared to the control group. Among them, *Gstm1* and *Gstyc2* were further and significantly up-regulated in the PBO + SGE group, as compared to the PBO group. Furthermore, cell cycle arrest and DNA repair-related genes, such as *Cdkn1b, Gadd45a,* and *Rad50* were significantly up-regulated by coadministration of SGE with PBO.

DISCUSSION

Chemical carcinogens that act by generating free radical metabolites are associated with many biochemical and molecular changes that induce oxidative stress leading to tumor promotion. In accordance with our previous

Table 4. Enzyme activities in livers of rats given PBO and/or SGE after DEN-initiation.

	No. of rats examined	TBARS levels (nmol MDA/g liver)	GST activity (nmol CDNB conjugate formed/min/mg protein)	GSH-px activity (nmol NADPH oxidized/min/mg protein)
Control	4	154.84 ± 21.18	77.2 ± 13.7	8.42 ± 0.59
PBO	6	228.62 ± 19.4**	141.39 ± 4.22**	9.52 ± 1.37
PBO+SGE	6	195.16 ± 11.31*, #	154.7 ± 12.69**, #	12.63 ± 2.36**, #

Results are mean ± S.D. for indicated number of animals for each group.
*,** $P < 0.05$ or $P < 0.01$vs. Control, respectively
PBO at $P < 0.05$ vs. PBO

Table 5. Microarray data: Up-regulated (> 1.5 fold) genes in the liver of a PBO + SGE-treated rat compared to a PBO-treated rat.

Function	Symbol	Description	Ratio (PBO+SGE/PBO)	Accession no.
Drug metabolizing enzymes				
	Gsr	Glutathione reductase	6.85	NM_053906
	Gpx2	Glutathione peroxidase 2	4.86	NM_183403
	Nqo1	NAD(P)H dehydrogenase, quinone 1	3.55	NM_017000
	Gstp2	Glutathione S-transferase, pi 2	5.09	NM_138974
	Gstm3	Glutathione S-transferase, mu type 3	2.83	NM_031154
	Gstt1	Glutathione S-transferase theta 1	2.72	NM_053293
	Ephx1	Epoxide hydrolase 1, microsomal	4.77	NM_012844
DNA repair				
	Rad50	RAD50 homolog (S. cerevisiae)	2.54	NM_022246
Cell cycle arrest				
	Gadd45a	Growth arrest and DNA-damage-inducible 45 alpha	5.81	NM_024127
	Cdkn1b	Cyclin-dependent kinase inhibitor 1B	3.88	NM_031762
Apoptosis				
	Prdx2	Peroxiredoxin 2	1.5	NM_017169
	Ddit3	DNA-damage inducible transcript 3	4.21	NM_024134

studies on mice and rats (Muguruma *et al.*, 2006, 2007), we successfully reproduced the tumor-promoting activity of PBO in response to oxidative stress. We also confirmed the role of lipid peroxidation in the tumor-promoting activity. A striking finding of the present study was that SGE coadministration inhibited lipid peroxidation. Such a decrease in lipid peroxidation by SGE was also reported in type 2 diabetic Goto-Kakizaki rats (Suzuki *et al.*, 2007). Antioxidants exert their action against oxidant-mediated damage by impairment of lipid peroxidation or due to their antioxidant activity (Shahjahan *et al.*, 2005). On the other hand, in the cell-free microsomal system in the present study, there was no marked difference in the ROS production between the PBO and PBO +SGE

Table 6. Real-time RT-PCR analysis of mRNA expression in liver tissues of the control, PBO and PBO + SGE groups.

Symbol	Control (n = 4)	PBO (n = 5)	PBO+SGE (n = 5)
Gpx2	1.29 ± 0.9	14.07 ± 2.03**	27.88 ± 4.48**, ##
Nqo1	1.09 ± 0.36	2.62 ± 0.7**	4.07 ± 0.43**, #
Gsr	1 ± 0.11	2.85 ± 0.45**	4.21 ± 0.52**, ##
Afar	1 ± 0.12	5.06 ± 1.7*	9.12 ± 1.72**, #
GST-yc2	1.34 ± 1.24	27.88 ± 10.91**	46.73 ± 11.07**, #
GST-m1	1 ± 0.09	22.62 ± 4.4**	33.11 ± 7.64**, #
GST-m3	1.02 ± 0.2	1.73 ± 0.22**	2 ± 0.21**
GST-a2	1.01 ± 0.18	1.91 ± 0.35**	2.44 ± 0.42**
Udpgtr2	1.01 ± 0.12	9.76 ± 2.34**	14.89 ± 1.53**, #
Abcc3	1.181 ± 0.74	22.19 ± 3.47**	37.6 ± 7.91**, #
Me1	1.01 ± 0.14	3.15 ± 0.81**	4.22 ± 0.42**, #
Gadd45a	1.05 ± 0.4	2.16 ± 0.66*	3.75 ± 0.9**, #
Cdkl1b	1.02 ± 0.25	1.76 ± 0.67	3.72 ± 1.04*, #
Rad50	1.15 ± 0.57	1.62 ± 0.43	3.06 ± 0.76**, #
Cyp1a1	1.59 ± 1.43	30.74 ± 12.5**	35.79 ± 14.04**
AhR	1.02 ± 0.22	1.93 ± 0.46**	1.87 ± 0.35**

Results are mean ± S.D. for indicated number of animals for each group.
*,** $P < 0.05$ or $P < 0.01$vs. Control, respectively.
#,## $P < 0.05$ or $P < 0.01$vs. PBO, respectively.

groups, suggesting that SGE was not associated with ROS generation during PBO metabolism through the induction of CYP1A1.

In the present study, mRNA levels of phase II drug-metabolizing enzyme genes, such as *Gpx2*, *Nqo1*, *Gsr*, *Udpgtr2*, *Afar*, *Gstm1*, and *Gstyc2*, were increased by coadministration of SGE with PBO. GST (GST-*m1* and GST-*m3*) and GSH-Px activities also increased in parallel to the changes in mRNA expression. *Gpx2*, *Nqo1*, *Gsr*, *Afar*, *Gstm1* and *Gstyc2* contain an antioxidant response element (ARE), i.e., a consensus sequence motif for the transcription factor Nrf 2, in their promoter regions. Also, the rat *Udpgtr2* gene is homologous to the mouse *Ugt2b1* gene that is also known to be regulated by Nrf 2 (Shelby and Klaassen, 2006). In addition, *Me1*, a NADPH-generating malic enzyme gene that is known to be flanked by ARE at the promoter region (Li *et al.*, 2002), was shown to be up-regulated by SGE coadministration in the present study. *Abcc3* is an ATP-binding cassette gene coding for a phase III enzyme that is known to function in the transport of organic anion-conjugating chemicals and play a role in hepatic detoxification and tissue-specific distribution; it was also shown to have been up-regulated by SGE coadministration. *Abcc3* has been shown to be induced by activators of the constitutive androstane receptor and an ARE (Cherrington *et al.*, 2002). Thus, the genes up-regulated by SGE administration that were examined here were all regulated directly by Nrf 2-mediated transcription or indirectly following transcriptional activation of Nrf 2-dependent genes. Many antioxidants exert their chemopreventive activity by inducing phase II enzymes (Kim *et al.*, 2006; Wu *et al.*, 2006; Lee *et al.*, 2007; Yates *et al.*, 2006). Therefore, the results of the present study suggest that SGE also exerts antioxidant effects through activation of an ARE.

Liver weights and the number of GST-P-positive foci remained unchanged by coadministration of SGE; however, SGE coadministration increased the area of the GST-

P-positive foci, suggesting an increase in the number of GST-P-positive cells per focus. On the other hand, the number of PCNA-positive cells was unaltered by SGE coadministration. If SGE has a tumor-promoting action in the liver, the number of GST-P positive foci and PCNA positive cells should also be increased. In this respect, it may be concluded that the action of SGE on the liver may not be related to its tumor-promoting activity.

In conclusion, the results of the present study suggest that SGE exerts antioxidant activity by inducing phase II enzymes through the Nrf 2-Keap1-ARE transcriptional machinery, although the antioxidant activity of SGE is neither effective nor sufficient for suppression of PBO-promoted early hepatocarcinogenic responses. However, it is noteworthy that several antioxidant genes were fluctuated by SGE treatment in the ROS-mediated hepatocarcinogenesis, and these findings are very important for the future clarification of mechanism of SGE antioxidant activity. Further studies are necessary to determine the conditions under which SGE effectively shows cancer-preventive activity.

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