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Original Article

Liver tumor promoting effect of orphenadrine in rats and its possible mechanism of action including CAR activation and oxidative stress

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ABSTRACT — Orphenadrine (ORPH), an anticholinergic agent, is a cytochrome P450 (CYP) 2B inducer. CYP2B inducers are known to have liver tumor-promoting effects in rats. In this study, we performed a rat two-stage liver carcinogenesis bioassay to examine the tumor-promoting effect of ORPH and to clarify its possible mechanism of action. Male rats were given a single intraperitoneal injection of *N*-diethylnitrosamine (DEN) as an initiation treatment. Two weeks after DEN administration, rats were fed a diet containing ORPH (0, 750, or 1,500 ppm) for 6 weeks. One week after the ORPH-administration rats were subjected to two-thirds partial hepatectomy for the acceleration of hepatocellular proliferation. The number and area of glutathione *S*-transferase placental form-positive foci significantly increased in the DEN-ORPH groups. Real-time RT-PCR revealed increased mRNA expression levels of *Cyp2b1/2*, *Mrp2* and *Cyclin D1* in the DEN-ORPH groups and of *Gpx2* and *Gstm3* in the DEN-High ORPH group. Microsomal reactive oxygen species (ROS) production and oxidative stress markers such as thiobarbituric acid-reactive substances and 8-hydroxydeoxyguanosine were increased in the DEN-High ORPH group. Immunohistochemically, constitutively active/androstane receptor (CAR) were clearly localized in the nuclei of hepatocytes in the DEN-ORPH groups. These results suggest that ORPH causes nuclear translocation of CAR resulting in the induction of the liver tumor-promoting activity. Furthermore, oxidative stress resulting from ROS production is also involved in the liver tumor-promoting activity of ORPH.

Key words: Orphenadrine, Cytochrome P450 2B inducer, Constitutive active/androstane receptor, Reactive oxygen species, Liver tumor, Rat

INTRODUCTION

Orphenadrine (ORPH), a derivative of the antihistamine diphenhydramine, is an anticholinergic agent that is antagonistic to the central and peripheral muscarinic receptors. It is used in the early stages of Parkinson's disease because of its skeletal muscle relaxant properties, and is also used as an analgesic in a variety of musculoskeletal conditions (Brocks, 1999). Since this agent has been approved as a drug in the 1950s, information on the genotoxicity study results is not available. On the other hand, ORPH induces cytochrome P450 family 2 subfamily B (CYP2B) in the livers of rats, and the induction of CYP2B is dependent on the nuclear translocation of the constitutive active/androstane receptor (CAR) (Murray *et al.*, 2003).

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Phenobarbital (PB), one of the CYP2B inducers, is a liver tumor promoter related to the nuclear translocation of CAR and to oxidative DNA damage resulting from the generation of microsomal reactive oxygen species (ROS). It has been reported that treatment with PB increased mRNA expression and protein levels of CYP2B in a two-stage liver carcinogenesis bioassay in rats (Kinoshita *et al.*, 2003; Waxman and Azaroff, 1992). The nuclear translocation of CAR induced by PB stimulates the expression of various genes that enhance hepatic tumor promotion (Deguchi *et al.*, 2009; Kawamoto *et al.*, 1999). In addition, it has been shown that microsomal ROS production and production of thiobarbituric acid-reactive substances (TBARS) and 8-hydroxydeoxyguanosine (8-OHdG) are induced by PB with increasing the *Cyp2b* (Morita *et al.*, 2011). Taking these facts into account, we hypothesize that ORPH has a liver tumor-promoting activity similar to the activity of PB.

In this study, we performed a two-stage liver carcinogenesis bioassay in rats to examine the hepatic tumor-promoting effect of ORPH and to clarify the possible mechanism of action, with a particular focus on the gene expression and biochemical events of ROS generation, and TBARS and 8-OHdG production in the liver. Additionally, we analyzed the localization of CAR protein expression in hepatocytes and the expression of CAR related-genes in the liver.

MATERIALS AND METHODS

Chemicals

Orphenadrine citrate salt (ORPH; CAS No.4682-36-4, purity: > 99%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). *N*-diethylnitrosamine (DEN; CAS No. 55-18-5, purity: > 99%) was purchased from Tokyo Kasei Kogyo (Tokyo, Japan).

Animals and experimental design

Animals received humane care according to the Guide for Animal Experimentation of the Tokyo University of Agriculture and Technology. Five-week-old male F344/N rats were purchased from Japan SLC, Inc. (Shizuoka, Japan). Rats were housed in cages on clean racks with up to 4 rats per cage, in an air-conditioned room with a 12-hr light/dark cycle (40-70% humidity, and 20-26°C temperature), and had free access to a basal diet (Oriental MF; Oriental Yeast Industries Co., Ltd., Tokyo, Japan) and tap water. After a one-week acclimatization period, a two-stage liver carcinogenesis model was performed as follows: Forty four animals were divided into four groups consisting of 8 (control group), 12 (DEN-alone group), 12 (DEN-Low ORPH group) and 12 (DEN-High ORPH group) animals. All animals except those in the control group received an intraperitoneal injection of 200 mg DEN/kg body weight dissolved in saline to initiate hepatocarcinogenesis. After 2 weeks on the basal diet and tap water, animals in the ORPH-treated groups were fed a diet containing either 750 or 1,500 ppm ORPH for 6 weeks. All animals were subjected to two-thirds partial hepatectomy for acceleration of hepatocellular proliferation 1 week after ORPH treatment. During the partial hepatectomy, one rat each in the DEN-alone and DEN-Low ORPH groups died because of technical errors. Two weeks after the partial hepatectomy one rat in the control group died. For selection of the ORPH dosage we referred to a previous report (Murray *et al.*, 2003). The highest dosage in our study was set at half of the dosage calculated from the report because of the taste of ORPH. At the end of the experiment, the rats were euthanized by exsanguination under ether anesthesia, and the livers were excised and weighed. Sliced liver samples were fixed in 4% phosphate-buffered paraformaldehyde for histopathology and immunohistochemistry. The remaining pieces of the livers were frozen in liquid nitrogen and stored at -80°C until further analysis.

Histopathology and immunohistochemistry

The fixed liver slices were washed with phosphate buffered saline, dehydrated with graded ethanol, embedded in paraffin, and sectioned for histopathological and immunohistochemical examinations. Hematoxylin and eosin (HE) staining was conducted according to routine histopathological methods. In addition, immunohistochemical staining of glutathione *S*-transferase placental form (GST-P), proliferating cell nuclear antigen (PCNA) and CAR was performed by the following procedure: Deparaffinized liver sections were treated with 0.3% H_2O_2 in methanol at room temperature for 30 min to block endogenous peroxidase, and then incubated overnight at 4°C with rabbit anti-GST-P antibody (1:1,000 dilution; Medical and Biological Laboratories Co., Ltd., Aichi, Japan), mouse anti-PCNA antibody (1:500 dilution; Dako, Glostrup, Denmark) or mouse anti-CAR1/2 antibody (1:50 dilution, Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The sections were heated by microwave at 90°C for 10 min for PCNA staining or autoclaved at 120°C for 100 sec for CAR1/2 staining, in 10 mmol/l citrate buffer (pH 6.0) before quenching the endogenous peroxidase activity. The avidin-biotin-peroxidase complex method (Vectastain Elite ABC system; Vector Laboratories, Burlingame, CA, USA) was then employed with 3,3-diaminobenzidine as a chromogen, followed by light counterstaining with hema-

toxylin. The number and areas of GST-P positive foci (> 0.2 mm diameter) and the total areas of the liver sections were measured using the WinRoof software (Mitani Corp., Fukui, Japan). The number of nuclei that were strongly positive for PCNA was counted for 10 random fields (approximately 700-900 hepatocytes in each field) per animal, and their % values were shown as the PCNA positive ratio.

cDNA microarray analysis

Total RNA was extracted with RNeasy Mini Kits (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Using 10 μg of total RNA from one animal each of the control, DEN-alone and DEN-High ORPH groups, double-stranded cDNA was synthesized with the Superscript Double-Stranded cDNA Synthesis kit (Invitrogen Corp., Carlsbad, CA, USA), according to the manufacturer's protocol. After labeling with Cy3, 6 μg of each Cy3-labeled cDNA sample was loaded onto the *Rattus norvegicus* Roche NimbleGen microarray for Gene Expression (Roche NimbleGen., Madison, WI, USA: Euk Expr 385K catalog Arr, 26,739 targets/microarray). Differentially expressed genes were analyzed using the Robust Multiple Average (RMA) normalization method (Irizarry *et al.*, 2003). Gene information was retrieved from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) websites.

Real-time reverse transcription polymerase chain reaction (RT-PCR) analysis

Expression of the genes listed in Table 1 was quantified using quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) analysis. Briefly, the total RNA from six rats in each group was extracted using RNeasy Mini Kits (Qiagen) according to the manufacturer's instructions. The total RNA was reversely transcribed by using ThermoScript reverse transcriptase (Super Script III First-Strand Synthesis System; Invitrogen). All PCR reactions were performed using the *Power* SYBR® Green PCR Master Mix (Applied Biosystems Japan Ltd., Tokyo, Japan) and were carried out using an Applied Biosystems StepOnePlus™ Real-Time PCR System (Applied Biosystems Japan Ltd.) under the following conditions: one cycle at 50°C for 2 min followed by 95°C for 10 min, and 40 cycles at 95°C for 15 sec and 60°C for 1 min. The forward and reverse primers listed in Table 1 were designed using the Primer Express 3.0 software following Applied Biosystems' instructions for optimal primer design. The relative differences in gene expression were calculated using the cycle time (Ct) values that were first normalized with those of actin beta, the endogenous control in the same sample, and then relative to a control Ct value by a $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001) using the StepOnePlus software (Applied Biosystems Japan Ltd.). The data are presented as the average fold changes with standard deviation.

Table 1. Primers used for real-time RT-PCR

Accession no.	Gene description	Gene symbol	Forward (5' → 3')	Reverse (5' → 3')
NM_012540	Cytochrome P450, family 1, subfamily a, polypeptide 1	*Cyp1a1*	gccttcacatcagccacaga	ttgtgactctaaccacccagaatc
NM_001198676	Cytochrome P450, family 2, subfamily b, polypeptide1and 2	*Cyp2b1/2*	gggacactgaaaaagagtgaagct	aatgccttcgccaagacaaat
NM_183403.2	Glutathione peroxidase 2	*Gpx2*	gtgtgatgtcaatgggcagaat	agggcagcttgtctttcaggta
NM_020540.1	Glutathione *S*-transferase mu 3	*Gstm3*	gaacgttcgcggacttactca	acgtatctcttctcctcatagtttgaatct
NM_022941	Nuclear receptor subfamily 1, group I, member 3	*Nr1i3 (CAR)*	catttccatgccctgacttgtg	aggctggacaatggcgtctc
NM_012833	ATP-binding cassette, subfamily C, member 2	*Abcc2 (Mrp2)*	gtggtccatacttttcatcatcctt	aagcactcagccagaggttagag
NM_171992	Cyclin D1	*Cyclin D1 (Ccnd1)*	cccacgatttcatcgaacact	gtgcatgtttgcggatgatct
NM_131906	Solute carrier organic anion transporter family, member 1a4	*Slco1a4 (Slc21a5)*	ggctctgcctgtctgagtacctt	ttgttaagcctgccactggaa
NM_031144	Actin, beta	*Actb*	ccctggctcctagcaccat	agagccaccaatccacacaga

Microsomal reactive oxygen species (ROS) production

Microsomal fractions were obtained according to the method of Yoshihara *et al.* (2001). Briefly, liver samples from six rats in each group were homogenized with three volumes of ice-cold 1.15% KCl-0.05 mol/l Tris-HCl buffer (pH 7.4) using TissueLyser II (Qiagen). The homogenate was centrifuged at 3,000 rpm for 10 min, and the supernatant was centrifuged at 11,000 rpm for 20 min. The resultant supernatant was further centrifuged at 51,000 rpm for 90 min. Finally, the pellet was resuspended in 1.15% KCl-0.05 mol/l Tris-HCl buffer (pH 7.4) as the microsomal fraction and stored at -80°C. Microsomal protein concentrations were determined by a BCA Protein Assay Kits (Pierce, IL, USA).

ROS was measured by the partially modified method of Serron *et al.* (2000). Microsomes (final concentration 0.2 mg/ml) were incubated in the dark at 37°C in 40 mM Tris buffer (pH 7.4) and 5 μM 2',7'-dichlorodihydro-fluorescein diacetate (H_2DCFHDA, Invitrogen). At the end of the incubation period, H_2O_2 (final concentration 0.1 mM) as a positive control or SKF-525A (final concentration 0.1 mM, Toronto Research Chemicals. North York, ON, Canada), a well-known inhibitor of cytochrome P450, were added, and the samples were further incubated at 37°C for 30 min in the dark. After that, nicotinamide adenine dinucleotide phosphate (NADPH; final concentration 0.5 mM; Oriental Yeast Co., Ltd., Tokyo, Japan) was added, and the rate at which ROS formed the fluorescent product was measured using a Synergy HT Multi-Detection Microplate Reader (BioTek, Winooski, VT, USA) with excitation and emission wavelengths of 485 and 528 nm, respectively. The data were normalized to control values expressed as 100%.

Determination of 8-OHdG and TBARS levels

Oxidative DNA damage and lipid peroxidation in the livers were estimated by the levels of 8-OHdG and TBARS, respectively.

The 8-OHdG level in liver DNA was determined in six liver samples from each of the DEN-alone and DEN-High ORPH groups using the method of Umemura *et al.* (2006). Briefly, nuclear DNA was isolated from 0.3 g of the wet weight samples using a DNA Extractor WB Kit (Wako Pure Chemical Industries., Osaka, Japan) containing an antioxidant NaI solution to dissolve the cellular components. For further prevention of autoxidation in the cell-lysis step, deferoxamine mesylate was added to the lysis buffer (Helbock *et al.*, 1998). The DNA was digested into deoxynucleotides with nuclease P1 and alkaline phosphatase. The levels of 8-OHdG (8-OHdG/10^5 deoxyguanosine) were then assessed by high-performance liquid chromatography with an electrochemical detection system (Coulochem II; ESA Biosciences, Inc., MA, USA) according to running conditions reported previously (Umemura *et al.*, 2006).

Hepatic TBARS levels were determined in six liver samples from each group using the method of Ohkawa *et al.* (1979). Briefly, 0.2 ml of liver homogenate was mixed with 1.15% KCl (18 mg/ml protein), 0.2 ml of 8.1% sodium dodecyl sulfate and 3.0 ml of 0.4% thiobarbituric acid in 10% acetic acid (pH 3.5), heated at 95°C for 60 min and then cooled. The reaction mixture was centrifuged at 4,000 rpm for 10 min after 1.0 ml of distilled water and 5.0 ml *n*-butanol and pyridine (15:1, v/v) were added. The absorbance of the resulting solution was determined spectrophotometrically at 532 nm using the Synergy HT Multi-Detection Microplate Reader (BioTek). The levels of TBARS were expressed as the equivalents of the malondialdehyde (MDA) amounts that were produced from 1,1,3,3-tetramethoxypropane.

Statistical analysis

All data are expressed as means with their standard deviations. Multigroups (Control, DEN-alone, DEN-Low ORPH and DEN-High ORPH groups) were used to test the homogeneity of variance between the groups by Bartlett's test. When the data were homogenous, Tukey's test was used, and when heterogeneous, Steel-Dwass test was used. A P value of less than 0.05 was considered statistically significant.

RESULTS

Body and liver weights, food and water consumption and estimated compound intake

During the experimental period, no clinical symptoms or deaths that might be related to the treatment were observed in either of the ORPH-treated groups. Body weight gains during the treatment period were significantly lower in the DEN-ORPH groups than in the control and DEN-alone groups (Fig. 1). Food consumption and water intake were significantly lower in the DEN-High ORPH group than in the control group (Table 2).

At necropsy, body weight was decreased dose-dependently by ORPH treatment. There were no significant differences in the absolute liver weights among the groups. Relative liver weights were significantly higher in the DEN-ORPH treated groups compared with the control and DEN-alone groups. The estimated daily intakes of ORPH in the DEN-Low ORPH and DEN-High ORPH groups calculated from total intakes were 40.4 and 73.9

Table 2. Body weight, food, and liver weight of rats given ORPH for 6 weeks after DEN initiation

Groups	control	DEN-alone	DEN-Low ORPH	DEN-High ORPH
Number of rats	7	11	11	12
Final body weight (g)	264.6 ± 12.3[a]	256.1 ± 12.2	245.1 ± 14.8*	210.3 ± 10.9**,##
Food intake (g/rat/day)[b]	13.0 ± 1.4	11.4 ± 0.7	11.9 ± 0.3	10.2 ± 0.1**
Total ORPH intake (mg/kg BW)			1805.0	3327.7
Water intake (g/rat/day)[b]	18.2 ± 1.4	18.0 ± 0.2	17.5 ± 0.8	13.2 ± 1.9*,##
Absolute liver weight (g)	7.1 ± 1.4	7.8 ± 0.8	8.0 ± 0.6	8.3 ± 0.6
Relative liver weight (% BW)	2.7 ± 0.5	3.0 ± 0.2	3.3 ± 0.1*,#	3.9 ± 0.2**,##

BW: Body weight.

[a]: Values are expressed as mean ± S.D.

[b]: Calculated from the last monitoring data.

*: $p < 0.05$, **: $p < 0.01$ significantly different from the control group (Tukey's test).

#: $p < 0.05$, ##: $p < 0.01$ significantly different from the DEN-alone group (Tukey's test).

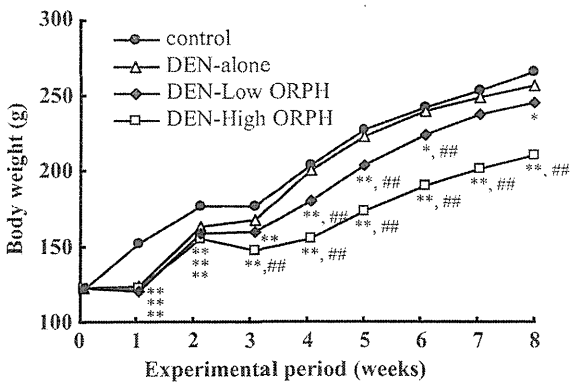

Fig. 1. Body weights of hepatectomized rats in control (black circles), DEN-alone (white triangles), DEN-Low ORPH (black diamonds), and DEN-High ORPH (white squares) groups. * $p < 0.05$, ** $p < 0.01$ significantly different from the control group (Tukey's test). ## $p < 0.01$ significantly different from the DEN-alone group (Tukey's test).

mg/kg body weight/day, respectively (Table 2), and these values were almost equal to the expected intake in the study.

Histopathological examinations

Histopathological examination of the liver samples showed centrilobular hepatocyte hypertrophy with eosinophilic cytoplasm and diffuse vacuolar degeneration in the ORPH treated groups (Fig. 2). Eosinophilic hepatocellular altered foci were also observed in the DEN-alone group and all of the ORPH treated groups.

GST-P positive foci, cell proliferation, and CAR protein expression in the liver

ORPH treatment significantly increased both the number and area of GST-P positive foci compared with the control and DEN-alone groups (Figs. 2 and 3). The PCNA-positive ratio was significantly higher in the DEN-ORPH groups compared with the control group (Figs. 2 and 3). Additionally, CAR was more clearly localized in the nuclei of liver cells in the DEN-ORPH groups compared with the DEN-alone group (Fig. 2).

cDNA microarray and real-time RT-PCR analyses

Hepatic gene expression changes were screened in one rat from each of the groups using an oligonucleotide microarray. In the CodeLink Bioarray, the expression of 392 genes showed a more than two-fold increase while 205 genes were decreased by more than a half in the rat of the DEN-High ORPH group as compared with the rat of the DEN-alone group (Supplemental data). Among the upregulated genes, we focused on the phase I drug-metabolizing enzymes, oxidative stress response-related genes, and CAR-related genes. Real-time RT-PCR analysis was performed on the genes listed in Table 1 in the livers of six rats per group (Table 3). Among the phase I drug-metabolizing enzymes, a significant increase was observed in the gene expression of *Cyp1a1* in the DEN-High ORPH rats and of *Cyp2b1/2* in all DEN-ORPH treated rats compared with the DEN-alone group. Furthermore, antioxidant and/or detoxifying genes against oxidative stress such as *Gpx2* and *Gstm3* were significantly higher in the DEN-High ORPH group compared with the DEN-alone group. In addition, the mRNA level of *Mrp2*, a CAR related-gene, was significantly high-

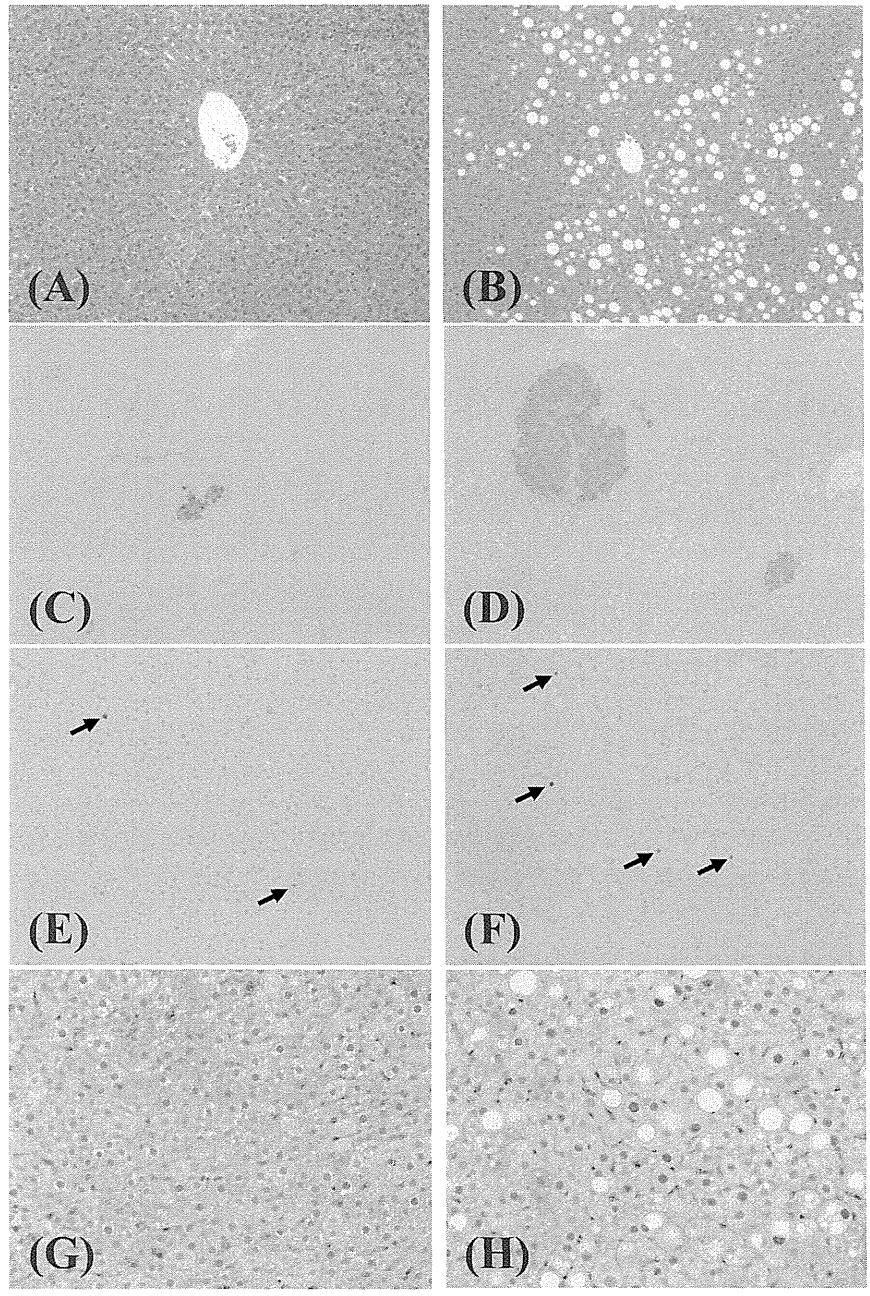

Fig. 2. Light microscope photographs of the livers of hepatectomized rats given ORPH for 6 weeks after DEN initiation. (A, B) HE staining. Original magnification: × 100. (C, D) GST-P immunostaining. Original magnification: × 40. (E, F) PCNA immunostaining. Original magnification: × 100. (G, H) CAR immunostaining. Original magnification: × 200. (E) Control group. (A, C, G) DEN-alone group. (H) DEN-Low ORPH group. (B, D, F) DEN-High ORPH group. (B) Diffuse hepatocellular hypertrophy and vacuolar degeneration are induced by 1,500 ppm ORPH treatment. (D) The area of GST-P positive foci is increased by ORPH treatment. (F) The number of PCNA positive cells (arrow) is increased by ORPH compared with control group (E). (H) CAR is more clearly localized in the nuclei of the DEN-Low ORPH group (H) compared with the DEN-alone group (G).

Table 3. mRNA expression in the livers of rats given ORPH for 6 weeks after DEN initiation

Groups	control	DEN-alone	DEN-Low ORPH	DEN-High ORPH
Number of rats examined	6	6	6	6
Gene name				
Cyp1a1	1.08 ± 0.44[a]	1.20 ± 0.59	1.49 ± 0.47	3.80 ± 2.89*,#
Cyp2b1/2	1.05 ± 0.32	1.18 ± 0.38	9.21 ± 3.51*,#	45.14 ± 15.28*,#
Gpx2	1.15 ± 0.78	1.94 ± 0.77	2.52 ± 0.85	6.18 ± 2.32*,#
Gstm3	1.06 ± 0.40	1.24 ± 0.55	1.85 ± 0.91	10.74 ± 6.17*,#
Nr1i3 (CAR)	1.05 ± 0.35	1.20 ± 0.37	1.18 ± 0.47	1.32 ± 0.24
Abcc2 (Mrp2)	1.01 ± 0.17	1.13 ± 0.27	1.38 ± 0.06*	1.59 ± 0.10*,#
Cyclin D1 (Ccnd1)	1.04 ± 0.36	2.03 ± 0.69*	3.57 ± 1.80*	15.61 ± 9.15*,#
Slco1a4 (Slc21a5)	1.03 ± 0.28	1.07 ± 0.25	0.91 ± 0.11	0.86 ± 0.11

[a]: Values of mRNA expression levels (normalized by actb) are expressed as mean ± S.D.
*: $p < 0.05$: significantly different from the control group (Tukey's test or Steel-Dwass test).
#: $p < 0.05$: significantly different from the DEN-alone group (Tukey's test or Steel-Dwass test).

er in all DEN-ORPH treated rats compared with the control group, and in the DEN-High ORPH group compared with the DEN-alone group. The mRNA level of *Cyclin D1*, a CAR related- and cell cycle regulated-gene, was significantly higher in the DEN-alone and ORPH treated groups compared with the control group, and in the DEN-High ORPH group compared with the DEN-alone group. The mRNA levels of *CAR* and *Slco1a4*, both of which are CAR related-genes, did not differ among the ORPH-treated groups and DEN-alone group. In the microarray analysis there were no interesting differences in gene expression among the rat from the DEN-alone group and the rat from the DEN-High ORPH group compared with the rat from the control group (date not shown).

Microsomal ROS production

To estimate the cellular sources of ROS, NADPH-dependent ROS production was measured in liver microsomes (Fig. 4). Without NADPH, no oxidized H_2DCF-DA, an indicator of ROS production, was observed in any of the groups. In contrast, ROS production was drastically increased by the addition of NADPH into the microsomal system. ROS production did not significantly differ between the control and DEN-alone groups but was significantly higher in the DEN-ORPH treated groups compared with the DEN-alone group. The addition of H_2O_2 or SKF-525A, a well-known inhibitor of CYP, increased ROS production (data not shown) or decreased ROS production, respectively, in each group (Fig. 4).

Oxidative stress on cellular membrane and DNA

To evaluate cellular oxidative damage caused by ROS production derived from CYP induction, hepatic TBARS formation and 8-OHdG content were determined (Fig. 4). The TBARS content was not significantly different between the control and DEN-alone groups, but was significantly higher in the DEN-High ORPH group compared with the DEN-alone group. Furthermore, the 8-OHdG content was significantly higher in the DEN-High ORPH group compared with the DEN-alone group (Fig. 4).

DISCUSSION

ORPH, a CYP2B inducer, is used in the early stages of Parkinson's disease in humans (Brocks, 1999), but no data are available on the genotoxicity and carcinogenicity of ORPH in experimental animals at present. In the present study, ORPH administration significantly increased the area and number of preneoplastic GST-P positive foci and the PCNA-positive ratio in hepatectomized rats in a DEN-initiated hepatocarcinogenesis model. These results strongly suggest that ORPH has a liver tumor-promoting activity in rats. In human, the dose for the treatment of Parkinson's disease is 200 mg/person, according to the manufacturer's prescription. It is 3.3 mg/kg body weight/day in case of person weighing 60 kg. This dose level is < 1/10 the intake level of ORPH in the DEN-low ORPH group (40.4 mg/kg body weight/day) in the present study. We recently found 375 ppm dietary concentration of ORPH is the threshold level that could not promote hepatocarcino-

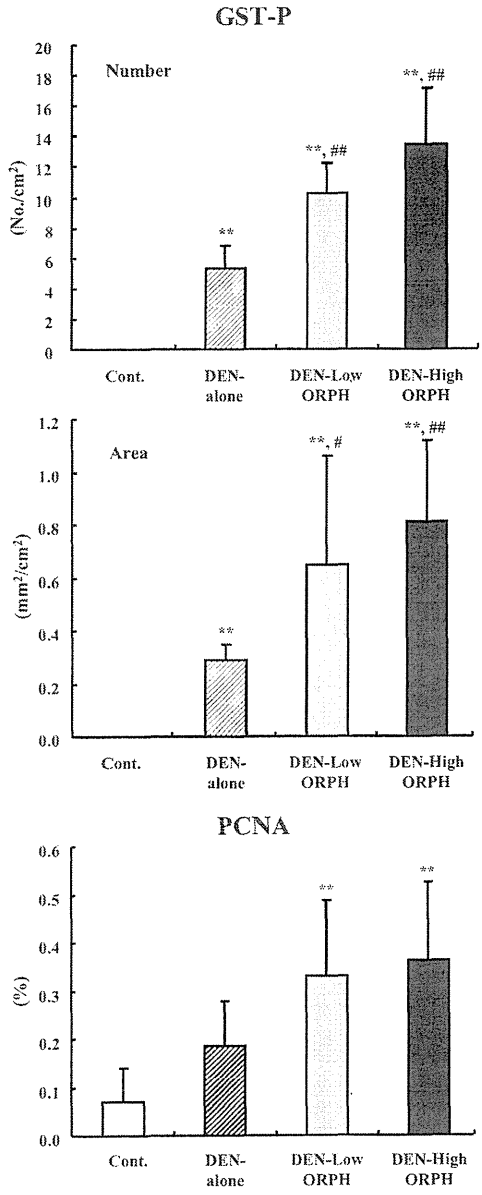

Fig. 3. Effect of ORPH treatment on GST-P positive foci and the PCNA-positive ratio in the livers of rats given ORPH for 6 weeks after DEN initiation. Columns represent the mean + S.D. ** $p < 0.01$ significantly different from the control group (Turkey's test or Steel-Dwass test). # $p < 0.05$, ## $p < 0.01$ significantly different from the DEN-alone group (Tukey's test or Steel-Dwass test).

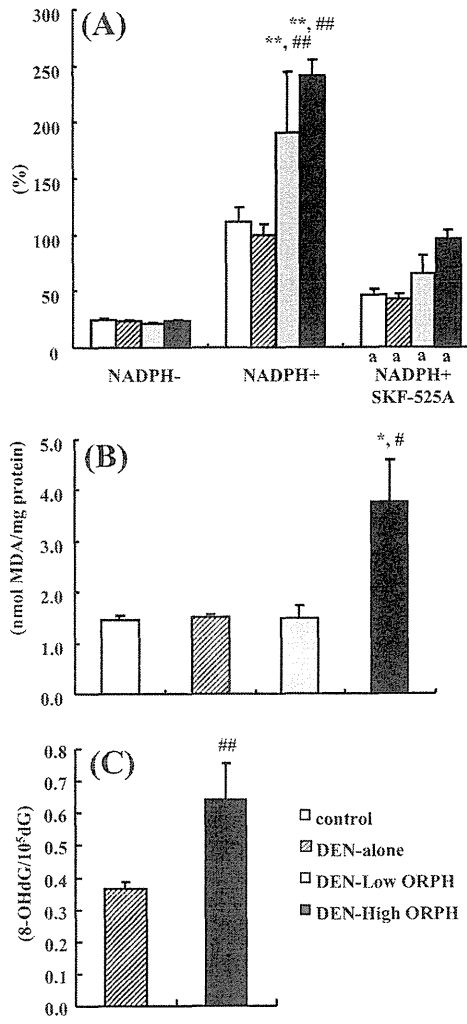

Fig. 4. Hepatic ROS production and TBARS and 8-OHdG expression in the control (white), DEN-alone (hatched), DEN-Low ORPH (gray) and DEN-High ORPH (black) groups. Each column represents the mean+S.D. (A) NADPH-dependent microsomal ROS production studied by the fluorescent probe H_2DCFDA. Left panel: without NADPH; middle panel: with NADPH; right panel: with NADPH and SKF-525A (a well-known inhibitor of CYP) in the pooled fraction. (B) TBARS levels. TBARS levels in the DEN-High ORPH are significantly higher than in the control and the DEN-alone groups. (C) 8-OHdG content. 8-OHdG content in the DEN-High ORPH group is significantly higher than in the DEN-alone group. * $p < 0.05$, ** $p < 0.01$ significantly different from the control group (Tukey's test or Steel-Dwass test). # $p < 0.05$, ## $p < 0.01$ significantly different from the DEN-alone group (Tukey's test or Steel-Dwass test). [a] significantly different from the production with NADPH (Tukey's test).

genesis (Reiko Morita and Kunitoshi Mitsumori, unpublished data). These results suggest that patients suffering from Parkinson's disease given clinical doses of ORPH may not promote hepatocarcinogenesis, while the contribution of genotoxic potential of this compound should be addressed on the hepatocarcinogenicity.

It has been shown that the chemicals that cause nuclear translocation of CAR and induce CYP2B have a liver tumor-promoting activity in rats (Deguchi *et al.*, 2009). Chang (2009) described that the CAR protein is a receptor for xenobiotic ligands and an inducer of CYP2B1 in rats. The CAR is localized in the cytoplasm in a complex with heat shock protein 90 (HSP90) and cytoplasmic CAR retention protein (CCRP). Once the CAR binds to an agonist, it is dissociated from HSP90 and CCRP, and the ligand-bound CAR translocates to the nucleus. In the nucleus, the CAR forms heterodimer with retinoid X receptor (RXR) (Honkakoshi *et al.*, 1998) and binds to DNA response elements in CAR target genes including *Cyp2b* (Sueyoshi *et al.*, 1999). In rats, *Cyp2b* is the most representative gene that is transcribed by CAR action (Honkakoshi *et al.*, 1998; Kawamoto *et al.*, 1999). Furthermore, CAR regulates not only the expression of various genes of *Cyp2b* but also of genes related to bioactivation, detoxification, and cell proliferation/tumorigenesis (Chang, 2009), such as *Cyclin D1*, one of the genes activated by CAR and promoting hepatocellular growth (Ledda-Columbano *et al.*, 2000). In addition, CAR is essential in the hepatic tumor-promotion mechanism (Deguchi *et al.*, 2009).

It has been known that the CAR is related to CYP2B induction of ORPH (Murray *et al.*, 2003). In the present study, immunohistochemistry revealed that nuclear translocation of CAR was increased by ORPH treatment. Although the mRNA levels of *CAR* and *Slco1a4*, a CAR related gene, were not significantly changed by ORPH treatment, the mRNA levels of *Mrp2* and *Cyclin D1*, among the CAR related genes (Maglich *et al.*, 2002; Ledda-Columbano *et al.*, 2000), were significantly higher in the ORPH-treated rats compared with the control group. These findings indicate that nuclear translocation of CAR is stimulated by ORPH treatment and suggest that ORPH increases the nuclear translocation of CAR, which results in the induction of liver tumor promotion in rats.

The CYP family generates ROS as byproducts of microsomal oxidation, and the upregulation of CYP1A1 and 1A2 isoforms indirectly results in the production of large amounts of ROS (Nishikawa *et al.*, 2002; Puntarulo *et al.*, 1998). Therefore, CYP1A inducers are known to generate large amount of ROS. In addition, it has been demonstrated that CYP1A inducers such as piperonyl butoxide, oxfendazole and β-naphthoflavone can generate ROS via a metabolic pathway in the livers of rats, which induces oxidative stress that is involved in hepatocarcinogenesis (Dewa *et al.*, 2009; Muguruma *et al.*, 2007). Similarly, PB, a CYP2B1/2 inducer, has been shown to increase hydroxyl radical levels in the livers of rats (Kinoshita *et al.*, 2002; Waxman *et al.*, 1992). Imaoka *et al.* (2004) reported that the induction of CYP2B1 by PB induces ROS production and also induces genomic DNA oxidation that may contribute to the initiation of liver tumors or tumor promotion by PB. In the present study, the mRNA levels of *Cyp1a1* and *Cyp2b1/2*, phase I drug-metabolizing enzymes, significantly increased by the administration of ORPH. The level of *Cyp1a1* was only increased in the DEN-High ORPH group and its induction ratio was low. On the other hand, the level of *Cyp2b1/2* was increased in all of the ORPH treated groups and its induction ratio was extremely high compared with that of *Cyp1a1*. These results suggest that ORPH mainly induces CYP2B during the metabolic process in the rat liver. It has been reported that chemicals that induce CYP2B and have a hepatic tumor promoting activity produce ROS during the metabolism of these, and the resulting oxidative stress stimulates the tumor promoting effect in the rat liver (Dewa *et al.*, 2009; Morita *et al.*, 2011). In the present study, ROS production was increased in rats given 750 ppm ORPH or more, and the level of TBARS and 8-OHdG was increased in the DEN-High ORPH group. The level of TBARS indicates the lipid peroxidation level in hepatocytes (Ohkawa *et al.*, 1979), and the increased level of TBARS in the DEN-High ORPH animals suggests that the overproduction of ROS resulting from the ORPH treatment caused high levels of oxidative stress in the liver. The 8-OHdG is a well-known oxidative guanine and a marker of oxidative damage in cellular components (Kasai *et al.*, 1986). There are more than 100 types of oxidative base modification in mammalian DNA (Croteau and Bohr, 1997), and 8-OHdG is one of the most abundant types of oxidative DNA damage (Cheng *et al.*, 1992). In addition, oxidative stress concomitant with increased 8-OHdG may have an important role in the liver tumor-promoting action of pentachlorophenol in mice (Umemura *et al.*, 2006). The results of our study and the above references suggest that 8-OHdG production resulting from the ROS generation may result in increased induction of preneoplastic lesions in the livers of rats given ORPH. We estimated 8-OHdG levels in the DEN-alone and the DEN-high ORPH groups, but not in the DEN-low ORPH group. Since 8-OHdG level is correlated with the TBARS level (Endoh *et al.*, 1996), we considered that

the 8-OHdG level in the DEN-low ORPH group may not show a remarkable increase as compared with the DEN-alone group, taking into account the data on TBARS in the DEN-low ORPH group.

Furthermore, the mRNA levels of *Gpx2* and *Gstm3*, phase II drug-metabolizing enzymes and antioxidative genes, were significantly increased in the DEN-High ORPH group. These antioxidative genes are regulated by Nrf2. Nrf2, which is a transcriptional factor, regulates the expression of genes for phase II drug-metabolizing enzymes by binding to the antioxidant responsive elements (AREs) (Itoh *et al.*, 1997), and is activated by oxidative/electrophile stress (Baird and Dinkova-Kostova, 2011). Nrf2 is present in the cytosol in a latent complex with the actin-anchored chaperone Kelch-like ECH-associated protein 1 (Keap1), a sulfhydryl-rich protein that is oxidized by oxidative/electrophile stress, thereby uncoupling the association between Nrf2 and Keap1. Subsequently, Nrf2 translocates to the nucleus, and associates with small Maf protein. The heterodimers bind to the AREs of target genes including *Gpx2* and *Gstm3* (Reisman *et al.*, 2009; Thimmulappa *et al.*, 2002). Glutathione *S*-transferases (GSTs), including *Gstm3,* detoxify xenobiotics by conjugating glutathione to a range of electrophilic substrates (Thimmulappa *et al.*, 2002). *Gpx2* is a member of the glutathione peroxidase (GPX) family and reduces H_2O_2 (Naiki-Ito, 2007). Upregulation of these genes suggests that ROS produced as a result of the ORPH treatment was probably eliminated by Nrf2 gene batteries. Therefore, it is unlikely that the ORPH treatment directly induces the alternation of these antioxidative enzyme expressions. However, as demonstrated by the increased levels of TBARS and 8-OHdG in the DEN-High ORPH group, it can be concluded that the excess ROS generation overcomes the functions of Nrf2-related antioxidative enzymes. Such an excess in ROS production and increased levels of TBARS and 8-OHdG strongly suggest the occurrence of "redox imbalance" in the rats given ORPH.

In conclusion, we have demonstrated that ORPH has liver tumor-promoting activity in rats. In addition, our results suggest that ORPH activates the CAR, resulting in the induction of the liver tumor-promoting activity. Furthermore, the high dosage of ORPH administration increased microsomal ROS production, subsequently inducing liver tumor-promoting effects in spite of an upregulation of antioxidative genes.

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Original Article

Enhanced liver tumor promotion activity in rats subjected to combined administration of phenobarbital and orphenadrine

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ABSTRACT — Phenobarbital (PB) and orphenadrine (ORPH) are cytochrome P450 (CYP) 2B inducers and have liver tumor-promoting effects in rats. In this study, we performed a rat two-stage liver carcinogenesis bioassay to examine the tumor-promoting effect of PB and ORPH co-administration. Twelve male rats per group were given an intraperitoneal injection of *N*-diethylnitrosamine (DEN) for initiation. Two-week after DEN administration, rats were given PB (60 or 120 ppm in drinking water), ORPH (750 or 1,500 ppm in diet) or 60 ppm PB+750 ppm ORPH for 6-week. One-week after the PB/ORPH treatment, all rats were subjected to two-thirds partial hepatectomy. To evaluate the effect of the combined administration, we used two statistical models: a heteroadditive model and an isoadditive model. In the heteroadditive model, the net values of the number and area of glutathione *S*-transferase placental form (GST-P) positive foci, *Cyp2b1/2*, *Gstm3* and *Gpx2* mRNA levels, microsomal reactive oxygen species (ROS) production and thiobarbituric acid-reactive substances level in the PB+ORPH group were significantly higher than the sum of the net values of those in the Low PB and Low ORPH groups. In the isoadditive model, the average values of the area of GST-P positive foci and PCNA positive hepatocyte ratio and *Gstm3* mRNA level in the PB+ORPH group were significantly higher than the average values of those in the High PB and High ORPH groups. These results suggest that PB and ORPH co-administration causes synergistic effects in liver tumor-promoting activity in rats resulting from oxidative stress due to enhanced microsomal ROS production.

Key words: Phenobarbital, Orphenadrine, CYP2B inducer, Reactive oxygen species, Liver tumor, Rat

INTRODUCTION

Phenobarbital (PB), a sedative and antiepileptic drug (Kwan and Brodie, 2004), is a liver tumor promoter in rats and representative non-genotoxic hepatocarcinogen (Feldman *et al.*, 1981). PB induces a large spectrum of drug metabolizing enzymes, including cytochrome P450 family 2 subfamily B (CYP2B) in the liver of rats (Kinoshita *et al.*, 2003; Waxman and Azaroff, 1992). In addition, it has been shown that microsomal reactive oxygen species (ROS) production and production of thiobarbituric acid-reactive substances (TBARS) induced with increasing *Cyp2b* induction due to PB treatment are involved in the liver tumor-promoting effect in rats (Morita *et al.*, 2011).

Orphenadrine (ORPH), a derivative of the antihistamine diphenhydramine, is an anticholinergic agent that is antagonistic to central and peripheral muscarinic receptors. It is used in the early stages of Parkinson's disease because of its skeletal muscle relaxant properties (Brocks, 1999). In contrast, it has been demonstrated that ORPH induces CYP2B in the livers of rats (Murray *et al.*, 2003) and has a liver tumor-promoting effect in rats attributable to oxidative DNA damage resulting from an increase in microsomal ROS production (Morita *et al.*, 2013).

It has been demonstrated that simultaneous administration of several hepatocarcinogens at doses lower than the

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apparent carcinogenic doses results in apparent carcinogenic actions in rats (Hasegawa *et al.*, 1989). This means that carcinogenic risk can be enhanced when subjects are simultaneously exposed to several hepatocarcinogens at doses lower than the individual hepatocarcinogenic dose. If simultaneous exposures to several hepatocarcinogens enhance their carcinogenic or tumor-promoting effects, we must reconsider the methods of risk evaluation to eliminate carcinogenic effects. We hypothesized that the combined administration of CYP2B inducers, PB and ORPH, enhances CYP2B induction resulting in greater ROS generation and intensifies the liver tumor promotion effect in rats.

In this study, we investigated the modifying effect of liver tumor promotion through combined administration of PB and ORPH in rats, with a particular focus on gene expression and biochemical events of ROS generation and TBARS in the liver.

MATERIALS AND METHODS

Chemicals

PB sodium (CAS No. 57-30-7, purity: > 98%) was purchased from Wako Pure Chemical Industries (Osaka, Japan). ORPH citrate salt (CAS No. 4682-36-4, purity: > 99%) and *N*-diethylnitrosamine (DEN; CAS No. 55-18-5, purity: > 99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Tokyo Kasei Kogyo (Tokyo, Japan), respectively.

Animals and experimental design

Animals received humane care according to the Guide for Animal Experimentation of the Tokyo University of Agriculture and Technology. Five-week-old male F344/N rats were purchased from Japan SLC, Inc. (Shizuoka, Japan). Rats were housed in cages on clean racks with up to 4 rats per cage, in an air-conditioned room with a 12-hr light/dark cycle (40-70% humidity and 20-26°C temperature) and had free access to a basal diet (Oriental MF; Oriental Yeast Industries Co., Ltd., Tokyo, Japan) and tap water. After a 1-week acclimatization period, a two-stage liver carcinogenesis model was performed as follows: 72 animals were divided into six groups consisting of 12 (DEN-alone group), 12 (Low PB group), 12 (High PB group), 12 (Low ORPH group), 12 (High ORPH group) and 12 (PB+ORPH group) animals. All animals received an intraperitoneal injection of 200 mg DEN/kg body weight dissolved in saline to initiate hepatocarcinogenesis. After 2 weeks, animals in the PB-treated groups received distilled water containing 60 ppm (Low PB and PB+ORPH groups) or 120 ppm (High PB group) PB, and ORPH-treated groups were fed a diet containing 750 ppm (Low ORPH and PB+ORPH groups) or 1,500 ppm (High ORPH group) ORPH for 6 weeks. All animals were subjected to two-thirds partial hepatectomy for acceleration of hepatocellular proliferation 1 week after PB or ORPH treatment. During the partial hepatectomy, one and two rats in the Low ORPH and DEN-alone groups, respectively, died because of technical errors. The threshold tumor promotion dose of PB and ORPH was set as the low dose and combination dose, and the twofold higher dose was set as the high dose in the present study. The threshold tumor promotion dose of PB and ORPH was determined as 60 ppm (Kitano *et al.*, 1998) and 750 ppm (based on our previous study; data not shown), respectively. Body weight, food intake and water intake were measured once a week, and the last measurement was done on the last day of the experiment. At the end of the experiment, the unfasted rats were euthanized by exsanguination under deep isoflurane anesthesia, and the livers were excised and weighed. Sliced liver samples were fixed in 4% phosphate-buffered paraformaldehyde for histopathology and immunohistochemistry. The remaining pieces of the livers were frozen in liquid nitrogen and stored at -80°C until further analysis.

Histopathology and immunohistochemistry

The fixed liver slices were washed with phosphate buffered saline, dehydrated with graded ethanol, embedded in paraffin, and sectioned for histopathological and immunohistochemical examinations. Hematoxylin and eosin (HE) staining was conducted according to routine histopathological methods. In addition, immunohistochemical staining of glutathione *S*-transferase placental form (GST-P) and proliferating cell nuclear antigen (PCNA) was performed by the following procedure: deparaffinized liver sections were treated with 0.3% H_2O_2 in methanol at room temperature for 30 min to block endogenous peroxidases and then incubated overnight at 4°C with rabbit anti-GST-P antibody (1:1,000 dilution; Medical and Biological Laboratories Co., Ltd., Aichi, Japan) or mouse anti-PCNA antibody (1:500 dilution; Dako, Glostrup, Denmark). The sections were heated in a hot water bath at 60°C for 20 min for PCNA staining in 10 mM citrate buffer (pH 6.0) before quenching the endogenous peroxidase activity. The avidin-biotin-peroxidase complex method (Vectastain Elite ABC system; Vector Laboratories, Burlingame, CA, USA) was then employed with 3,3-diaminobenzidine as a chromogen, followed by light counterstaining with hematoxylin. The number and areas of GST-P positive foci (≥ 0.2 mm diameter) and the total areas of the liver sections were measured using the Win-

Roof software (Mitani Corp., Fukui, Japan). The number of nuclei that were strongly positive for PCNA was counted for 10 random fields (approximately 700-900 hepatocytes in each field) per animal, and their % values are shown as the PCNA positive ratio.

Real-time reverse transcription polymerase chain reaction (RT-PCR) analysis

Expression levels of the genes listed in Table 1 were quantified using quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) analysis. Briefly, the total RNA from six rats in each group was extracted using RNeasy Mini Kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The total RNA was reversely transcribed using by ThermoScript reverse transcriptase (Super Script III First-Strand Synthesis System; Invitrogen Corp., Carlsbad, CA, USA). All PCR reactions were performed using *Power* SYBR® Green PCR Master Mix (Applied Biosystems Japan Ltd., Tokyo, Japan) using an Applied Biosystems StepOnePlus™ Real-Time PCR System (Applied Biosystems Japan Ltd.) under the following conditions: one cycle at 50°C for 2 min followed by 95°C for 10 min and 40 cycles at 95°C for 15 sec and 60°C for 1 min. The forward and reverse primers listed in Table 1 were designed using the Primer Express 3.0 software following Applied Biosystems' instructions for optimal primer design. The relative differences in gene expression were calculated using the cycle time (Ct) values that were first normalized with those of beta actin, the endogenous control in the same sample and then relative to a control Ct value by the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001) using StepOnePlus software (Applied Biosystems Japan Ltd.). The data are presented as average fold changes with standard deviation.

Microsomal reactive oxygen species (ROS) production

Microsomal fractions were obtained according to the method of Yoshihara *et al.* (2001). Briefly, liver samples from six rats in each group were homogenized with three volumes of ice-cold 1.15% KCl-0.05 M Tris-HCl buffer (pH 7.4) using TissueLyser II (Qiagen). The homogenate was centrifuged at 3,000 rpm for 10 min, and the supernatant was centrifuged at 11,000 rpm for 20 min. The resultant supernatant was further centrifuged at 51,000 rpm for 90 min. Finally, the pellet was resuspended in 1.15% KCl-0.05 M Tris-HCl buffer (pH 7.4) as the microsomal fraction and stored at -80°C. Microsomal protein concentrations were determined by a BCA Protein Assay Kits (Pierce, IL, USA).

ROS was measured by the partially modified method of Serron *et al.* (2000). Microsomes (final concentration 0.2 mg/ml) were incubated in the dark at 37°C in 40 mM Tris buffer (pH 7.4) and 5 μM 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFHDA, Invitrogen). At the end of the incubation period, H_2O_2 (final concentration 0.1 mM) and a positive control or SKF-525A (final concentration 0.1 mM, Toronto Research Chemicals, North York, ON, Canada), a cytochrome P450 inhibitor, were added, and the samples were further incubated at 37°C for 30 min in the dark. After that, nicotinamide adenine dinucleotide phosphate (NADPH; final concentration 0.5 mM; Oriental Yeast Co., Ltd., Tokyo, Japan) was added, and the rate at which ROS formed the fluorescent product was measured using a Synergy HT Multi-Detection Microplate Reader (BioTek, Winooski, VT, USA) with excitation and emission wavelengths of 485 and 528 nm, respectively. The data were normalized to control values expressed as 100%.

Table 1. Primers used for real-time RT-PCR

Accession no.	Gene description	Gene symbol	Forward (5' → 3')	Reverse (5' → 3')
NM_012540	Cytochrome P450, family 1, subfamily a, polypeptide 1	*Cyp1a1*	gccttcacatcagccacaga	ttgtgactctaaccacccagaatc
NM_001198676	Cytochrome P450, family 2, subfamily b, polypeptide 1 and 2	*Cyp2b1/2*	gggacactgaaaaagagtgaagct	aatgccttcgccaagacaaat
NM_183403.2	Glutathione peroxidase 2	*Gpx2*	gtgtgatgtcaatgggcagaat	agggcagcttgtctttcaggta
NM_020540.1	Glutathione S-transferase mu 3	*Gstm3*	gaacgttcgcggacttactca	acgtatctcttctcctcatagtttgaatct
NM_031144	Actin, beta	*Actb*	ccctggctcctagcaccat	agagccaccaatccacacaga

Lipid peroxidation level

Lipid peroxidation in the livers was estimated by the level of TBARS. Hepatic TBARS level was determined in six liver samples from each group using the method of Ohkawa *et al.* (1979). Briefly, 0.2 ml of liver homogenate was mixed with 1.15% KCl (17.8 mg protein /ml), 0.2 ml of 8.1% sodium dodecyl sulfate and 3.0 ml of 0.4% thiobarbituric acid in 10% acetic acid (pH 3.5), heated at 95°C for 60 min and then cooled. The reaction mixture was centrifuged at 4,000 rpm for 10 min after 1.0 ml of distilled water and 2.0 ml *n*-butanol and pyridine (15:1, v/v) were added. The absorbance of the resulting solution was determined spectrophotometrically at 532 nm using the Synergy HT Multi-Detection Microplate Reader (BioTek). The levels of TBARS were expressed as equivalents of the malondialdehyde (MDA) amounts that were produced from 1,1,3,3-tetramethoxypropane.

Statistical analysis

All data are expressed as the mean ± standard deviation. Multiple groups (DEN-alone, Low PB, High PB, Low ORPH, High ORPH and PB+ORPH groups) were used to test homogeneity of variance between the groups by Bartlett's test. When the data were homogenous, Dunnett's test was used, and when heterogeneous, Steel test was used to compare with the DEN-alone or PB+ORPH group. In addition, to estimate the modifying effect of the combined administration, two different statistical analyses, a heteroadditive model and an isoadditive model, were performed using the method recommended by Futakuchi *et al.* (1996) and Hasegawa *et al.* (1991). A P value of less than 0.05 was considered statistically significant.

RESULTS

Body and liver weights, food and water consumption and estimated compound intake

During the experimental period, no clinical symptoms or deaths that might be related to the treatment were observed in either of the PB- and/or ORPH-treated groups. Body weight gain during the treatment period and final body weight were significantly lower in the High ORPH group than in the DEN-alone group (Table 2). Food intake and water intake were significantly lower in the High ORPH group than in the DEN-alone group (Table 2).

At necropsy, the absolute liver weights in the PB-treated groups were significantly higher than that in the DEN-alone group, and the absolute liver weight in the PB+ORPH group significantly increased compared to that in the Low/High ORPH groups. Relative liver weights in the Low/High PB, High ORPH and PB+ORPH groups significantly increased compared with the DEN-alone group, and the relative liver weight in the PB+ORPH group was significantly higher than that in the Low/High PB or Low ORPH groups (Table 2). The estimated total compound intakes of the PB- and/or ORPH-treated groups are shown in Table 2. There was little difference in the intake of PB between the PB+ORPH and Low PB groups,

Table 2. Body weight, food intake, water intake, total PB intake, total ORPH intake, liver weights and PCNA-positive hepatocyte ratio of rats given PB and/or ORPH for 6 weeks after DEN initiation

Groups	DEN-alone	Low PB	High PB	Low ORPH	High ORPH	PB+ORPH
Number of rats	10	12	12	11	12	12
Final body weight (g)	257.0 ± 19.3[a]	271.0 ± 16.8	264.7 ± 18.2[#]	241.7 ± 13.0	205.1 ± 23.3[**,##]	251.4 ± 11.5
Food intake (g/rat/day)[b]	13.3 ± 0.5	14.1 ± 0.5	13.4 ± 0.8	12.3 ± 0.7	10.8 ± 0.7[**]	12.3 ± 0.2
Water intake (g/rat/day)[b]	20.9 ± 1.4	20.9 ± 0.3	20.2 ± 0.6	19.9 ± 1.5	16.8 ± 1.9[**]	18.2 ± 0.5
Total PB intake (mg/kg BW)	-	243.4	447.8	-	-	221.8
Total ORPH intake (mg/kg BW)	-	-	-	1797.1	3405.4	1746.1
Absolute liver weight (g)	8.1 ± 0.6[##]	9.5 ± 0.8[**]	9.6 ± 0.9[**]	8.0 ± 0.7[##]	8.1 ± 1.2[##]	10.1 ± 0.8[**]
Relative liver weight (% BW)	3.1 ± 0.0[##]	3.5 ± 0.2[**,##]	3.6 ± 0.2[**,##]	3.3 ± 0.2[##]	3.9 ± 0.2[**]	4.0 ± 0.2[**]
PCNA positive hepatocyte ratio (%)	0.12 ± 0.05[#]	0.19 ± 0.21	0.29 ± 0.07[*,#]	0.23 ± 0.08[#]	0.42 ± 0.13[*]	0.51 ± 0.17[*]

[a] Values are expressed as the mean ± S.D.

[b] Calculated from the last monitoring data.

[*] $p < 0.05$, [**] $p < 0.01$ significantly different from the DEN-alone group (Dunnett's test or Steel test).

[#] $p < 0.05$, [##] $p < 0.01$ significantly different from the PB+ORPH group (Dunnett's test or Steel test).

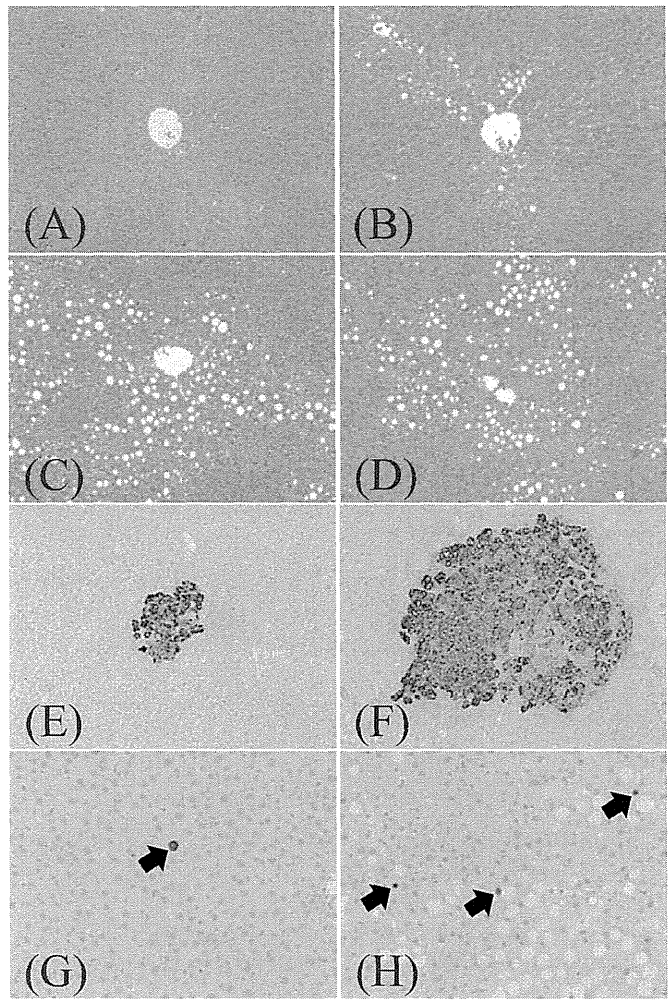

Fig. 1. Light microscope photographs of the livers in hepatectomized rats given PB and/or ORPH for 6 weeks after DEN initiation. (A-D) HE staining. Original magnification: x100. (E, F) GST-P immunostaining. Original magnification: x100. (G, H) PCNA immunostaining. Original magnification: x200. (A, E, G) DEN-alone group. (B) High PB group. (C) High ORPH group. (D, F, H) PB+ORPH group. (B, C, D) Diffuse hepatocellular hypertrophy and vacuolar degeneration induced by PB and/or ORPH treatment. (F) The area of GST-P positive foci enhanced by combined administration of PB and ORPH. (H) The number of PCNA positive cells (arrow) enhanced by the combined administration of PB and ORPH.

and no difference in the intake of ORPH was observed between the PB+ORPH and Low ORPH groups.

Histopathological examinations

Histopathological examinations of the liver samples showed centrilobular hepatocyte hypertrophy with eosinophilic cytoplasm and diffuse vacuolar degeneration in the PB- and/or ORPH-treated groups (Fig. 1). Eosinophilic hepatocellular altered foci were also observed in the High PB, High ORPH and PB+ORPH groups.

GST-P positive foci and cell proliferation in the liver

The number of GST-P positive foci in the High PB, High ORPH and PB+ORPH groups significantly increased compared with the DEN-alone group, and the number of GST-P positive foci in the PB+ORPH group significantly increased compared with the Low PB and Low ORPH groups. The area of GST-P positive foci in the PB+ORPH group significantly increased compared with the DEN-alone, Low/High PB or Low ORPH groups (Figs. 1 and

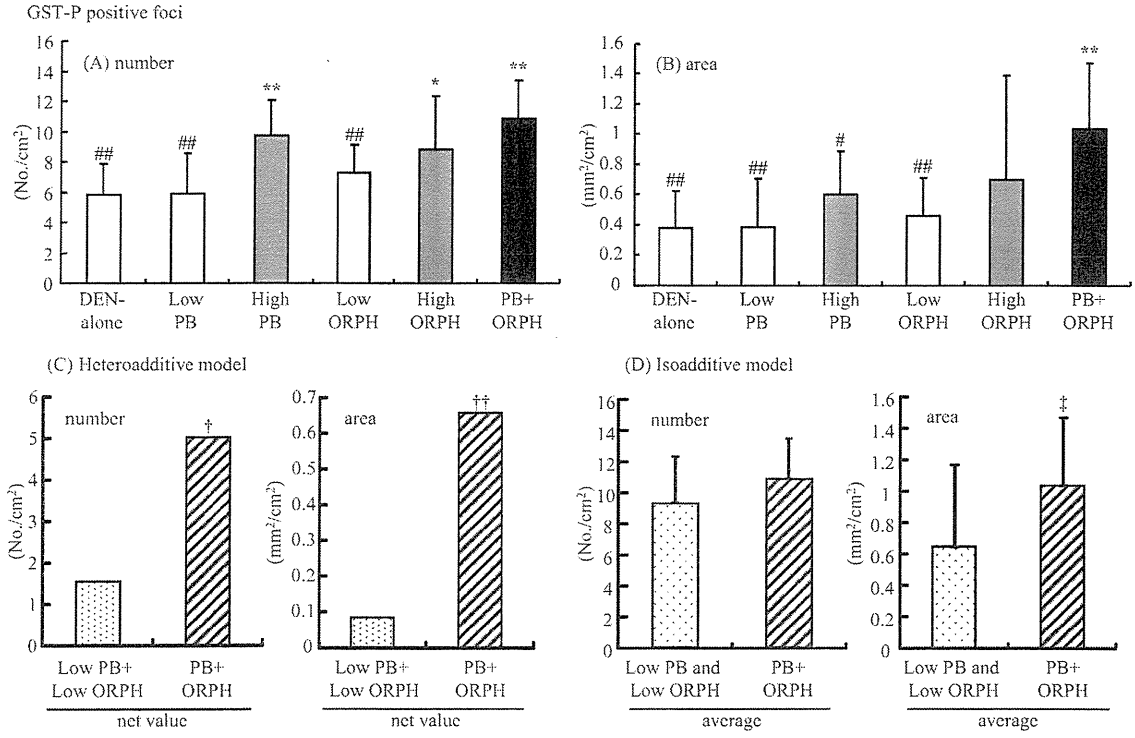

Fig. 2. Effects of PB and/or ORPH treatment on GST-P positive foci in the livers of rats given PB and/or ORPH for 6 weeks after DEN initiation. (A) The number of GST-P positive foci in each group. Columns represent the mean + S.D. (B) The area of GST-P positive foci in each group. * $p < 0.05$, ** $p < 0.01$: significantly different from the DEN-alone group (Dunnett's test or Steel test). # $p < 0.05$, ## $p < 0.01$: significantly different from the PB+ORPH group (Dunnett's test or Steel test). (C) The net values of number and area of GST-P positive foci in the PB+ORPH group (hatched) and the sum of the net values of the Low PB and Low ORPH groups (dot) in the heteroadditive model († $p < 0.05$, †† $p < 0.01$). (D) The average values of area of GST-P positive foci in the PB+ORPH group (hatched) and the average value of the High PB and High ORPH groups (dot) in the isoadditive model (‡ $p < 0.05$, *t*-test).

2). In the heteroadditive model, we obtained the net value by subtracting the control value (DEN-alone group). The net value of the combination at a low dose (number, 5.03 number/cm²; area, 0.66 mm²/cm²) was significantly higher (number, $p < 0.05$; area, $p < 0.01$) than the sum of the net value of both PB and ORPH at low doses (number, 1.55 number/cm²; area, 0.08 mm²/cm²) (Fig. 2). In the isoadditive model, the area of GST-P positive foci was significantly higher in the PB+ORPH group; the value of the combined administration of low doses of PB and ORPH (area, 1.04 ± 0.43 mm²/cm²) was significantly higher ($p < 0.05$, *t*-test) than the average value of each individual treatment at a high dose of PB and ORPH (area, 0.65 ± 0.52 mm²/cm²) (Fig. 2).

The PCNA positive hepatocyte ratio in the High PB, High ORPH and PB+ORPH groups was significantly higher than that in the DEN-alone group, and the PCNA positive hepatocyte ratio in the PB+ORPH group significantly increased compared with the High PB or Low ORPH groups (Table 2). In the heteroadditive model, there was no significant increase in the PCNA positive ratio in the PB+ORPH group, but the value of the PB+ORPH group (0.51%) in the isoadditive model was significantly higher ($p < 0.05$, *t*-test) than the average value of the individual treatment at a high dose of PB and ORPH (0.35%).

Real-time RT-PCR analysis

Real-time RT-PCR analysis was performed on the genes listed in Table 1 in the livers of six rats per group (Table 3). Among the phase I drug-metabolizing enzymes, the gene expression levels of *Cyp1a1* in the ORPH-treated groups and *Cyp2b1/2* in the PB- and/or ORPH-treated groups significantly increased compared with the DEN-alone group, and the gene expression of *Cyp2b1/2* in the PB+ORPH group was higher than that in the Low PB or Low ORPH groups. In the heteroadditive model, a synergistic effect in *Cyp2b1/2* induction was observed in the PB+ORPH group (the net value of the PB+ORPH group, 77.91 folds; the sum of the net value of the low dose groups, 51.74 folds; $p < 0.01$), but not in the isoadditive model.

Furthermore, antioxidant and/or detoxifying genes against oxidative stress, such as *Gstm3* and *Gpx2*, were significantly higher in the Low/High PB, High ORPH and PB+ORPH groups than those in the DEN-alone group. Additionally, in the PB+ORPH group, *Gstm3* significantly increased compared with levels in the Low PB or Low ORPH groups, and *Gpx2* significantly increased compared with levels in the Low ORPH group. In the heteroadditive model, the net value of *Gstm3* in the PB+ORPH group (9.31-fold) was significantly higher than the sum of the net value of that in the low dose groups (3.71-fold; $p < 0.01$). In the isoadditive model, the value of *Gstm3* in the PB+ORPH group (10.33 ± 4.95-fold) was significantly higher than the average value of that in each individual treatment at a high dose of PB and ORPH (7.53 ± 6.98-fold; $p < 0.05$, *t*-test). In addition, there was a synergistic increase in *Gpx2* in the PB+ORPH group in the heteroadditive model, but not in the isoadditive model. The net value of the PB+ORPH group (1.61-fold) was significantly higher than the sum of the net value of the low dose groups (0.81-fold; $p < 0.05$).

Microsomal ROS production

To estimate the cellular sources of ROS, NADPH-dependent ROS production was measured in liver microsomes (Table 4). Without NADPH, no oxidized H_2DCF-DA, an indicator of ROS production, was observed in any of the groups. In contrast, ROS production drastically increased by the addition of NADPH into the microsomal system. ROS production in the PB- and/or ORPH-treated groups was significantly higher than that in the DEN-alone group, and the ROS production in the PB+ORPH group was significantly higher than that in the Low/High PB or Low ORPH groups. The addition of SKF-525A, a CYP inhibitor, decreased ROS production in each group. Additionally, the addition of H_2O_2 increased ROS production in each group (data not shown). In the heteroadditive model, the levels of ROS (net value: 117.91%) in the PB+ORPH group were significantly higher than the sum of the net value of the low dose groups (net value: 110.84%; $p < 0.01$). However, in the isoadditive model, there was no synergistic enhancement of ROS levels in the PB+ORPH group.

Oxidative stress on cellular membrane

To evaluate cellular oxidative damage caused by ROS production derived from CYP induction, hepatic TBARS formation was determined (Table 4). The TBARS content in the High ORPH and PB+ORPH groups was significantly higher than that in the DEN-alone group, and the TBARS content in the PB+ORPH group was significantly higher than that in the Low/High PB or Low ORPH groups. In the heteroadditive model, the levels of TBARS (net value: 0.45 nmol MDA/mg protein) in the PB+ORPH group were significantly higher than the sum of the net value of the low dose groups (net value: 0.07 nmol MDA/mg protein; $p < 0.01$). However, in the isoadditive model, there was no synergistic enhancement in TBARS levels in the PB+ORPH group.

Table 3. mRNA expression in the livers of rats given PB and/or ORPH for 6 weeks after DEN initiation

Groups	DEN-alone	Low PB	High PB	Low ORPH	High ORPH	PB+ORPH
Number of rats examined	6	6	6	6	6	6
Gene name						
Cyp1a1	1.06 ± 0.35[a,#]	2.27 ± 1.02	2.57 ± 2.04	2.83 ± 1.37*	16.64 ± 4.68*,#	4.71 ± 2.74*
Cyp2b1/2	1.04 ± 0.31#	38.94 ± 11.56*,#	63.78 ± 13.24*	14.88 ± 13.07*,#	75.71 ± 14.61*	78.96 ± 11.54*
Gstm3	1.02 ± 0.21#	3.94 ± 1.31*,#	6.44 ± 2.37*	1.81 ± 0.44#	8.61 ± 2.63*	10.32 ± 2.22*
Gpx2	1.02 ± 0.22#	1.83 ± 0.54*	2.49 ± 0.59*	1.02 ± 0.19#	3.02 ± 0.91*	2.63 ± 0.76*

[a] Values of mRNA expression levels (normalized by *actb*) are expressed as the mean ± S.D.

* $p < 0.05$ significantly different from the DEN-alone group (Steel test).

# $p < 0.05$ significantly different from the PB+ORPH group (Steel test).

Table 4. TBARS level and microsomal ROS production of rats given PB and/or ORPH for 6 weeks after DEN initiation

Groups	DEN-alone	Low PB	High PB	Low ORPH	High ORPH	PB+ORPH
Number of rats	6	6	6	6	6	6
Microsomal ROS production (%)						
-NADPH	28.70 ± 1.85[a]	28.45 ± 2.63	26.62 ± 1.31	26.62 ± 1.31	26.08 ± 1.77	24.99 ± 1.87
+NADPH	100.00 ± 8.09[##]	144.06 ± 16.69[**,##]	181.08 ± 31.49[**,#]	166.78 ± 25.64[**,##]	234.86 ± 14.17[**]	217.91 ± 26.76[**]
+NADPH+SKF-525A	39.68 ± 2.20[†]	52.37 ± 8.24[†]	63.22 ± 6.30[†]	49.01 ± 11.30[†]	83.21 ± 6.94[†]	69.66 ± 8.86[†]
TBARS level (nmol MDA/mg protein)	1.25 ± 0.18[##]	1.15 ± 0.16[##]	1.31 ± 0.15[#]	1.29 ± 0.21[#]	1.69 ± 0.16[*]	1.70 ± 0.37[**]

[a] Values are expressed as the mean ± S.D.

[*] $p < 0.05$, [**] $p < 0.01$ significantly different from the DEN-alone group (Dunnett's or Steel test).

[#] $p < 0.05$, [##] $p < 0.01$ significantly different from the PB+ORPH group (Dunnett's or Steel test).

[†] significantly different from the production with NADPH (*t*-test)

DISCUSSION

In the present study, the combined administration of low doses of PB and ORPH significantly increased the number of GST-P positive foci compared with the Low PB or Low ORPH group, and the area of GST-P positive foci in the PB+ORPH group was significantly higher than that in the Low/High PB or Low ORPH groups. However, it is unclear whether such an enhancing effect of GST-P positive foci in the PB+ORPH group is synergistic or additive. It has been reported that the combined administration of hepatocarcinogens, such as 2-AAF, benzo[a]pyrene and N-ethyl-N-hydroxyethylnitrosamine at low doses, resulted in synergistic effects in the rat liver (Hasegawa *et al.*, 1989). In addition, Hasegawa *et al.* (1994, 1995) used two statistical models, the heteroadditive model and the isoadditive model. In the isoadditive model, they compared the average value of the individual treatments of the carcinogenic basic dose and the value of ten HCAs and the combination treatment at 1/10 dose of the basic dose. As a result, a significant increase was not shown in the combination group in the isoadditive model. Therefore, they concluded that this combination effect was not synergistic. Futakuchi *et al.* (1996) examined potential synergism between four N-nitroso compounds in rat liver carcinogenesis in a medium-term bioassay. In their report, the effect value of the individual chemicals was obtained by subtracting the control value (DEN-alone group). In the heteroadditive model, they compared the sum of the effect values of each individual treatment at 1/4 dose and the value of rats treated with four chemicals in combination at the 1/4 dose level, and no significant increase was observed between them. However, in the isoadditive model, the value in combination at the 1/4 dose level treatment was almost equivalent to the average of four individual treatments at full dose, and the value in combination at 1/16 dose level treatment was not significantly higher than the average value of four individual treatment at 1/4 dose. They concluded that the combined effects were not synergistic but isoadditive. Taking into account the above reports, we have also adopted the same statistical analysis for the data obtained in the present study. In the heteroadditive model, the net value of the combination at a low dose (number and area of GST-P positive foci) was significantly higher than the sum of the net values of both PB and ORPH at a low dose (Fig. 2). In the isoadditive model, the area of GST-P positive foci was significantly higher in the PB+ORPH group than the average value of each individual treatment at a high dose of PB and ORPH (Fig. 2). Furthermore, the PCNA positive hepatocyte ratio in the PB+ORPH group significantly increased compared with the High PB or Low ORPH groups. In the heteroadditive model, there was no significant increase in the PCNA positive ratio in the PB+ORPH group, but the value of the PB+ORPH group in the isoadditive model was significantly higher than the average value of the individual treatment at a high dose of PB and ORPH. These results strongly suggest that the combined liver tumor promotion effect of PB and ORPH is synergistic in rats.

The CYP family generates ROS as byproducts of microsomal oxidation, and PB, a CYP2B1/2 inducer, has been shown to increase hydroxyl radical levels in the livers of rats (Kinoshita *et al.*, 2003; Waxman and Azaroff, 1992). Imaoka *et al.* (2004) reported that CYP2B1 induction by PB induces ROS production and genomic DNA oxidation that may contribute to tumor promotion by PB. ORPH, a CYP2B inducer, is used in the early stages of Parkinson's disease in humans (Brocks, 1999), though no data are available on the genotoxicity and carcino-