INTRODUCTION

Cytochrome P450 (CYP) is a metabolic enzyme that acts on various xenobiotics (Nebert et al., 2000). It is generally accepted that the microsomal electron system, including CYP and nicotinamide adenine dinucleotide phosphate (NADPH)-CYP450 reductase, generates reactive oxygen species (ROS) via metabolism (Puntarulo and Cederbaum, 1998). Many CYP1A inducers are found in industrial chemicals and are known to have hepato-cellular tumor-promoting activities in rodents, such as β-naphthoflavone (BNF), oxfendazole (OX), and omeprazole (OPZ) (Deva et al., 2008, 2009; Hayashi et al., 2012a). Many of these chemicals induce ROS generation in the microsomal fractions, and this ROS generation is suspected to enhance the liver tumor promotion by altering cellular physiological functions, such as oxidative proteins, lipid peroxidation, DNA damage, and cell signaling (Deva et al., 2008, 2009). Indole-3-carbinol (I3C) is an alkaloid contained in cruciferous vegetables, such as broccoli, Brussels sprouts, cabbage, and cauliflower (Higdon et al., 2007), and also has been shown to increase CYP1A mRNA expression in the liver of rats (Nho and Jeffery, 2001; Coe et al., 2006). Our group previously reported that I3C promoted hepatocellular tumors in parallel with the CYP1A induction potential to cause subsequent oxidative stress responses in rats (Shimamoto et al., 2011a, 2011b).

CYP2B is associated with xenobiotic detoxification, and the CYP2B subfamily is responsible for the metab-
olism of more than 25% of commercial drugs in humans (Martignoni et al., 2006). Phenobarbital (PB), a sedative and antiepileptic drug, induces a large spectrum of drug-metabolizing enzymes, including CYP2B in the liver of rats (Waxman and Azaroff, 1992; Kinoshita et al., 2003), and is known as a CYP2B inducer. PB is well known as a liver tumor promoter in rats and a representative non-genotoxic hepatocarcinogen (Feldman et al., 1981; Kwan and Brodie, 2004). Morita et al. (2011) recently reported that production of microsomal ROS and thiobarbituric acid-reactive substances (TBARS) with increased Cyp2b induction resulting from PB treatment is involved in the effects of liver tumor promotion in rats. Orphenadrine (ORPH), a derivative of the antihistamine diphenhydramine, also 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 increased microsomal ROS production (Morita et al., 2013).

It has been demonstrated that co-administration of several hepatocarcinogens at doses lower than the apparent carcinogenic doses results in carcinogenic actions in rats (Hasegawa et al., 1989). Our group previously reported the modifying effect of liver tumor promotion by co-administration of CYP inducers in rats (Hayashi et al., 2012b; Morita et al., 2013). Hayashi et al. (2012b) reported that co-administration of CYP1A inducers, OPZ and BNF, resulted in synergistic effects in liver tumor promotion probably because of increased COX-2 expression. Co-administration of CYP2B inducers, PB and ORPH, also caused synergistic effects in liver tumor promotion resulting from oxidative stress following enhanced microsomal ROS production (Morita et al., 2013). Therefore, we hypothesized that the combined administration of CYP1A and CYP2B inducers also enhances CYP inductions resulting in oxidative stress and enhances liver tumor promotion in rats. In this study, we investigated the modifying effects of liver tumor promotion through co-administration of I3C and PB in rats, with a particular focus on gene expression and biochemical events of ROS generation and TBARS in the liver.

MATERIALS AND METHODS

Chemicals
Indole-3-carbinol (I3C; CAS No. 700-06-1) and phenobarbital (PB; CAS No. 57-30-7, purity: > 98%) were purchased from Wako Pure Chemical Industries (Osaka, Japan). N-diethyl-N-nitrosamine (DEN; CAS No. 55-18-5, purity: > 99%) was purchased from Tokyo Kasei Kogyo (Tokyo, Japan).

Animals
Five-week-old male F344 rats were purchased from Japan SLC, Inc. (Shizuoka, Japan). Rats were housed in cages on clean racks with up to four animals 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. Animals received humane care according to the Guide for Animal Experimentation of the Tokyo University of Agriculture and Technology.

Experimental design
After a 1-week acclimatization period, a two-stage liver carcinogenesis model was used as follows. Seventy-two rats were divided into six groups consisting of 12 animals each: DEN-alone group, Low PB group, High PB group, Low I3C group, High I3C group, and PB+I3C group. First, all rats were given an intraperitoneal injection of DEN (200 mg/kg body weight, dissolved in saline) to initiate hepatocarcinogenesis. Animals in the PB-treated groups received distilled water containing 60 ppm (Low PB and PB+I3C groups) or 120 ppm (High PB group) PB, and I3C-treated groups were fed a diet containing 2,500 ppm (Low I3C and PB+I3C groups) or 5,000 ppm (High I3C group) I3C for 6 weeks, starting 2 weeks after DEN initiation. The threshold tumor promotion dose of PB and I3C was set as the low dose and combination dose, and the two-fold higher dose was set as the high dose in the present study. The threshold tumor promotion doses of PB and I3C were determined as 60 ppm (Kitano et al., 1998) and 2,500 ppm (Shimamoto et al., 2011b), respectively. To enhance hepatocyte proliferation, all rats underwent a two-thirds partial hepatectomy (PH) 1 week after the start of the treatment of PB or I3C. Body weight, food, and water consumption were measured once a week, and the last measurement was done on the last day of the experiment. At the end of the experiment, rats were euthanized by exsanguination under deep isoflurane anesthesia, and livers were excised and weighed. Sliced liver samples were fixed in 4% phosphate-buffered paraformaldehyde (pH 7.4) 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
Fixed liver slices were washed with phosphate-buffered saline, dehydrated in graded ethanol, and embedded in paraffin. Sections were mounted onto glass slides and stained with hematoxylin and eosin (HE) or used for
immunohistochemical analysis. HE staining was conducted according to routine histopathological methods. Immunohistochemistry was performed by the avidin-biotin-peroxidase complex technique utilizing a Vectastain® Elite ABC Kit (Vector Laboratories Inc., Burlingame, CA, USA). Primary antibodies used were the anti-glutathione S-transferase placental form (GST-P) rabbit polyclonal antibody (1:1,000 dilution; Medical and Biological Laboratories Co., Ltd., Aichi, Japan). Sections were treated with 0.3% H2O2 in methanol for 30 min to remove endogenous peroxidase. Each section was incubated with the primary antibody at 4°C for 16 hr, with the secondary antibodies against mouse or rabbit IgG at room temperature for 30 min, and with avidin-biotin-peroxidase complex at room temperature for 30 min. Then, sections were developed in 0.05% 3,3’-diaminobenzidine/H2O2 as the chromogen. All immunostained slides were counterstained with hematoxylin. The number and areas of GST-P+ foci (≥0.2 mm diameter) and the total areas of the liver sections were measured using WinRoof software (Mitani Corp., Fukui, Japan).

To evaluate the cell proliferation, double-immunohistochemistry was performed with Ki-67 that is a nuclear protein associated with cellular proliferation (Gerdes et al., 1983) in combination with GST-P. Similar to the procedure described above, sections were incubated with anti-Ki-67 mouse monoclonal antibody (clone: MIB5, 1:50 dilution, Dako, Glostrup, Denmark), followed by autoclaving at 121°C for 20 min in 10 mM citrate buffer (pH 6.0). Then sections were incubated with anti-GST-P antibody after which the second antigen detection was performed with Vectastain® ABC-AP kit (Vector Laboratories). Positive reactions appeared red after staining with Vector® Red (Vector Laboratories). All immunostained slides were counterstained with hematoxylin. The number of nuclei that were strongly positive for Ki-67 was counted under 400 × in 10 randomly selected fields (inside or outside the GST-P+ foci) per animal, and the % values are shown as the Ki-67+ ratio.

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

Total RNA from six samples per group was extracted using RNeasy Mini Kits (Qiagen, Hilden, Germany) and was reversely transcribed using thermostable reverse transcriptase (SuperScript® III First-Strand Synthesis System; Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s protocol. Quantitative real-time reverse-transcription-PCR with Power SYBR® Green PCR Master Mix (Life Technologies) was performed using Applied Biosystems StepOnePlus™ Real-Time PCR System. The forward and reverse PCR primers were designed using the Primer Express software (Version 3.0; Life Technologies). 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−ΔΔCT method (Livak and Schmittgen, 2001) using StepOnePlus software (Applied Biosystems Japan Ltd.). Primer sequences are shown in Table 1.

Microsomal ROS production

Microsomal fractions were obtained according to the method of Yoshihara et al. (2001). Briefly, liver samples from six rats per group were homogenized with three volumes of ice-cold 11.5% KCl-0.05 M Tris-HCl buffer

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

<table>
<thead>
<tr>
<th>Accession no.</th>
<th>Gene description</th>
<th>Gene symbol</th>
<th>Forward (5’ → 3’)</th>
<th>Reverse (5’ → 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_012540</td>
<td>Cytochrome P450, family 1, subfamily a, polypeptide 1</td>
<td>Cyp1a1</td>
<td>gccttcacatcagcagaaaga</td>
<td>ttgtaacatcagcacagaatc</td>
</tr>
<tr>
<td>NM_001198676</td>
<td>Cytochrome P450, family 2, subfamily b, polypeptide 1 and 2</td>
<td>Cyp2b1/2</td>
<td>gggcaactgaaagaaatggagat</td>
<td>agctgacactgaagcaata</td>
</tr>
<tr>
<td>NM_013105</td>
<td>Cytochrome P450, family 3, subfamily a, polypeptide 1</td>
<td>Cyp3a1</td>
<td>acagcagcacactctggaggt</td>
<td>ctctctctcagttctgtgta</td>
</tr>
<tr>
<td>NM_017000</td>
<td>NAD(P)H dehydrogenase, quinone 1</td>
<td>Nqo1</td>
<td>tcgccgagccactctg</td>
<td>ttgctggtggccaaataca</td>
</tr>
<tr>
<td>NM_020540.1</td>
<td>Glutathione S-transferase mu 3</td>
<td>Gstm3</td>
<td>gaacgtaggggactaactga</td>
<td>acgtctctctctctctcatgagctt</td>
</tr>
<tr>
<td>NM_183403.2</td>
<td>Glutathione peroxidase 2</td>
<td>Gpx2</td>
<td>gtctctgtaatgggcagaatg</td>
<td>agggagctgtgtctcaggt</td>
</tr>
<tr>
<td>NM_021835</td>
<td>Jun proto-oncogene</td>
<td>Jun</td>
<td>ctgcaagatggagacactt</td>
<td>ccacacagactgaggagaa</td>
</tr>
<tr>
<td>NM_001105720</td>
<td>Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha</td>
<td>Nfkbia</td>
<td>gtctagcccccagagcctt</td>
<td>aattactgatcccttccccaaat</td>
</tr>
<tr>
<td>NM_031144</td>
<td>Actin, beta</td>
<td>Actb</td>
<td>cctgtgctctactcagacet</td>
<td>agggacaccaacctcagcagag</td>
</tr>
</tbody>
</table>
(pH 7.4) using TissueLyser II (Qiagen). The homogenate was centrifuged at 700 × g, 4°C for 10 min, and the supernatant was centrifuged at 10,000 × g, 4°C for 20 min. The resultant supernatant was further centrifuged at 105,000 × g, 4°C for 90 min. Finally, the pellet was resuspended in 11.5% KCl-0.05 M Tris-HCl buffer (pH 7.4) as a microsomal fraction and stored at -80°C. The concentration of microsomal protein was determined using BCA Protein Assay Kits (Pierce, IL, USA).

ROS were 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 (H2DCFDA, Life Technologies). At the end of the incubation period, H2O2 (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 CYP inhibitor, were added, and the samples were further incubated at 37°C for 30 min in the dark. After that, NADPH (final concentration 0.5 mM; Oriental Yeast Industries Co., Ltd.) was added, and the rate at which ROS formed the fluorescent product was measured using a 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.

Lipid peroxidation levels

Lipid peroxidation in the liver was estimated by the level of TBARS. Hepatic TBARS levels were determined using six liver samples per group by the method of Ohkawa et al. (1979) with a minor modification. Briefly, 0.2 ml of liver homogenate was mixed with 1.15% KCl (17.8 mg protein/ml), 0.2 ml of 8.1% SDS, 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 2,070 × g for 10 min after addition of 1.0 ml of distilled water and 2.0 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). 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 were expressed as the mean ± standard deviation. Multiple groups (DEN-alone, Low PB, High PB, Low I3C, High I3C, and PB+I3C groups) were used to test homogeneity of variance between the groups by Bartlett’s test. If the variance was homogenous, numerical data were assessed using the Dunnett’s multiple comparison test, and if a significant difference in variance was observed, the Steel test was used instead.

In addition, to estimate the modifying effect of the com-

| Table 2. Final body weights, food and water consumption, PB intake, I3C intake, liver weights, and Ki-67+ cell ratio in the livers of male F344 rats given PB and/or I3C for 6 weeks after DEN initiation |
|---------------------------------|--------|--------|--------|--------|--------|--------|--------|
| Group                          | DEN-alone | Low PB | High PB | Low I3C | High I3C | PB+I3C |
| Number of animals examined     | 12     | 12     | 10     | 10     | 9      | 11     |
| Final body weight (g)          | 280.0 ± 13.2 | 280.7 ± 8.5 | 278.0 ± 20.1 | 273.3 ± 13.1 | 253.9 ± 16.5** | 264.5 ± 18.7 |
| Food consumption (g/rat/day)²  | 12.9 ± 0.1 | 12.3 ± 0.5 | 12.0 ± 0.1 | 12.8 ± 0.6 | 12.8 ± 1.6 | 12.4 ± 1.0 |
| Water consumption (g/rat/day)² | 18.9 ± 0.8 | 17.4 ± 0.6 | 15.9 ± 1.6 | 17.7 ± 0.7 | 19.5 ± 3.9 | 16.5 ± 1.6 |
| PB intake (mg/kg body weight/day) | –      | 4.2 ± 0.2 | 7.7 ± 0.9 | –      | –      | 3.9 ± 1.3 |
| I3C intake (mg/kg body weight/day) | –      | –      | –      | 1361.0 ± 128.3 | 2748.0 ± 571.9 | 1319 ± 65.5 |
| Liver weight (g)               | 9.1 ± 0.4⁽*⁾ | 9.4 ± 0.5⁽*⁾ | 10.6 ± 0.6⁽**⁾ | 12.0 ± 0.8⁽**⁾ | 11.9 ± 1.1⁽**⁾ | 11.4 ± 0.9⁽**⁾ |
| (% body weight)                | 3.2 ± 0.1⁽**⁾ | 3.3 ± 0.1⁽**⁾ | 3.5 ± 0.2⁽**⁾ | 4.3 ± 0.7⁽**⁾ | 5.0 ± 0.3⁽**⁾ | 4.3 ± 0.4⁽**⁾ |
| Ki-67⁺ cell ratio (%)          | 3.17 ± 1.19⁽**⁾ | 3.68 ± 1.13⁽**⁾ | 4.39 ± 0.83⁽**⁾ | 5.08 ± 0.86⁽**⁾ | 7.40 ± 1.38⁽**⁾ | 6.50 ± 1.09⁽**⁾ |
| Outside the GST-P⁺ foci        | 7.61 ± 2.81 | 6.90 ± 2.30 | 6.49 ± 2.12 | 9.73 ± 3.69 | 9.48 ± 1.16 | 8.33 ± 2.97 |
| Inside the GST-P⁺ foci         | 3.17 ± 1.19⁽**⁾ | 3.68 ± 1.13⁽**⁾ | 4.39 ± 0.83⁽**⁾ | 5.08 ± 0.86⁽**⁾ | 7.40 ± 1.38⁽**⁾ | 6.50 ± 1.09⁽**⁾ |

Values are expressed as mean ± S.D.

⁽*⁾ Calculated from the data obtained at the final sacrifice.

⁽²⁾ 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+I3C group (Dunnett’s test or Steel test).
combined administration, two different statistical analyses, a heteroa
dditive model and an isoadditive model, were performed using the metho
d recommended by Hasegawa et al. (1991) and Futakuchi et al. (1996).
In these models, a P value of less than 0.05 was considered statistically sig
ificant. It has been accepted that the dose response curve is very impo
rtant for the analysis of the combined effects and the isoadditive model
developed taking into account the dose response curve is more persuasive than the het-
eroadditive model that are not taken into the account the dose response curves (Reif et al., 1984).
Therefore, when a significant fluctuation was observed in the isoadditive model rather than the heteroadditive model, it was regarded that such a fluctuation is biologically significant.

RESULTS

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

During the experimental period, four rats died in the course of PH and four rats died after the PH because of technical errors, while 64 rats survived (12, 12, 10, 10, 9, and 11 rats in the DEN-alone, Low PB, High PB, Low I3C, High I3C, and PB+I3C groups, respectively). No signi
ificant differences in food and water consumption were found between the treated and DEN-alone groups, but the final body weight in the High I3C group was significantly lower than the DEN-alone group (Table 2).

The absolute and relative liver weights at necropsy significantly increased in the High PB, Low/High I3C and PB+I3C groups when compared with the DEN-alone group. The absolute and relative liver weights in the PB+I3C group were significantly higher than the DEN-alone and Low/High PB groups, and lower than the High I3C group (Table 2).

Estimated compound intakes of PB and I3C are shown in Table 2. No difference in the intakes of PB and I3C was observed between the PB+I3C group and Low PB or Low I3C group.

Histopathology, GST-P+ foci, and cell proliferation in the liver

Histopathologically, treatment with PB or I3C induced diffuse hepatocellular hypertrophy with eosinophilic cyto
plasm. Altered foci that have cellular alterations with eosino
philic hepatocytes distinguishable from normal hepatocytes and diffuse vacuolar degeneration were observed in the PB- and/or I3C-treated groups, and treatment with PB showed cytoplasmic vacuolar degeneration (Fig. 1). There was no inflammatory change in the PB- and/or I3C-treated groups.

Immunohistochemical analysis revealed that the number of GST-P+ foci significantly increased in the PB- and/or I3C-treated groups, and the areas of GST-P+ foci significantly increased in the Low/High I3C groups when compared with the DEN-alone group. The number of GST-P+ foci in the PB+I3C group was significant-
ly higher than the DEN-alone and the Low PB groups. The areas of GST-P+ foci in the PB+I3C group were significantly higher than the DEN-alone and the Low/ High PB groups (Fig. 2). These results were further ana
lyzed by two different statistical models, the heteroad

Table 3. Real-time RT-PCR analysis on the liver tissues from male F344 rats given PB and/or I3C for 6 weeks after DEN initiation

<table>
<thead>
<tr>
<th>Group</th>
<th>DEN-alone</th>
<th>Low PB</th>
<th>High PB</th>
<th>Low I3C</th>
<th>High I3C</th>
<th>PB+I3C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of animals examined</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Cyp1a1</td>
<td>1.09 ± 0.49*</td>
<td>2.17 ± 1.00*</td>
<td>2.12 ± 0.97*</td>
<td>5415.1 ± 563.85*</td>
<td>7453.8 ± 1167.65*</td>
<td>5915.9 ± 803.55*</td>
</tr>
<tr>
<td>Cyp2b1/2</td>
<td>1.18 ± 0.84**</td>
<td>17.55 ± 2.94**</td>
<td>23.29 ± 2.12**</td>
<td>20.82 ± 2.22**</td>
<td>28.20 ± 3.76**</td>
<td>22.03 ± 2.30**</td>
</tr>
<tr>
<td>Cyp3a1/2</td>
<td>1.02 ± 0.23**</td>
<td>1.75 ± 0.24**</td>
<td>4.58 ± 1.99**</td>
<td>4.40 ± 1.42*</td>
<td>6.24 ± 1.23*</td>
<td>5.91 ± 1.06*</td>
</tr>
<tr>
<td>Nqo1</td>
<td>1.01 ± 0.143*</td>
<td>1.36 ± 0.22*</td>
<td>3.19 ± 1.93*</td>
<td>10.74 ± 4.43*</td>
<td>11.14 ± 2.94*</td>
<td>16.41 ± 7.33*</td>
</tr>
<tr>
<td>Gstm3</td>
<td>1.07 ± 0.36*</td>
<td>4.58 ± 1.55**</td>
<td>9.20 ± 4.19*</td>
<td>12.48 ± 5.69*</td>
<td>26.97 ± 5.36*</td>
<td>19.39 ± 7.15*</td>
</tr>
<tr>
<td>Gpx2</td>
<td>1.04 ± 0.31**</td>
<td>2.33 ± 0.54**</td>
<td>5.07 ± 2.08**</td>
<td>12.12 ± 3.26*</td>
<td>23.22 ± 5.41*</td>
<td>15.70 ± 4.80*</td>
</tr>
<tr>
<td>Jun</td>
<td>1.06 ± 0.41**</td>
<td>1.00 ± 0.21*</td>
<td>1.18 ± 0.39*</td>
<td>1.74 ± 0.71</td>
<td>2.22 ± 0.66*</td>
<td>1.95 ± 0.46*</td>
</tr>
<tr>
<td>Nkb1a</td>
<td>1.06 ± 0.37</td>
<td>1.10 ± 0.25</td>
<td>1.06 ± 0.52</td>
<td>1.30 ± 0.87</td>
<td>1.01 ± 0.47</td>
<td>1.59 ± 0.35</td>
</tr>
</tbody>
</table>

Values (mRNA expression levels: normalized by Actb) are expressed as mean ± S.D.
* 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+I3C group (Dunnett’s test or Steel test).

Co-administration effect of I3C and PB on liver tumor promotion
To clarify the synergistic or additive effects of the interaction, the heteroadditive model was used. In this model, the area of GST-P+ foci between the net value obtained by subtracting the value of the DEN-alone group from the combination of PB and I3C at low doses (0.44 mm²/cm²) and the sum of the net values of both I3C and PB at low doses (0.50 mm²/cm²) showed no significant differences. However, the net value of the number of GST-P+ foci obtained by subtracting the value of the DEN-alone group from the combination of PB and I3C at low doses (11.52 number/cm²) was significantly lower (p < 0.05) than the sum of the net values of both I3C and PB at low doses (17.89 number/cm²). In the isoadditive model, there were no differences in the numbers and area of GST-P+ foci between the combined administration of low doses of I3C and PB at a high dose (number, 18.50 ± 5.84 number/cm²; area, 0.78 ± 0.21 mm²/cm²) and the average value of each individual treatment at a high dose of I3C and PB (number, 17.51 ± 5.85 number/cm²; area, 0.63 ± 0.24 mm²/cm²; Fig. 3A).

At the outside of the GST-P+ foci, the Ki-67+ cell ratio significantly increased in the Low/High I3C and PB+I3C groups when compared with the DEN-alone group. Furthermore, the Ki-67+ cell ratio in the PB+I3C group was significantly higher than the DEN-alone, Low/High PB, and Low I3C groups (Table 2). When the results were analyzed by the two different additive statistical models, there were no significant differences in the Ki-67+ cell ratio in the PB+I3C group in the additive statistical models. At the inside of the GST-P+ foci, no differences were observed between the treated and DEN-alone groups (Table 2). On the contrary, a suppression of the Ki-67+ cell ratio was observed in the PB+I3C group (the net value of the PB+I3C group, 0.72%; the sum of the net value of the low-dose groups, 1.41%; p < 0.05) in the heteroadditive model, but not in the iso-

![Fig. 1. Histopathological appearance of the livers in hepatectomized rats given PB and/or I3C for 6 weeks after DEN initiation. (A) DEN-alone group. (B) Low PB group. (C) Low I3C group. (D) PB+I3C group. HE stain. Original magnification: 100 ×. (B) Diffuse hepatocellular hypertrophy and vacuolar degeneration. (C, D) Altered hepatocellular foci composed of hepatocytes with eosinophilic cytoplasm.](image-url)
additive model (the value of the combined administration of low doses of I3C and PB, 8.56 ± 2.97%; the average value of each individual treatment at a high dose of I3C or PB, 7.91 ± 2.28%).

Real-time RT-PCR analyses

In real-time RT-PCR analyses, the gene expression levels of phase I drug-metabolizing enzymes (Cyp1a1, Cyp2b1/2, Cyp3a1, Nqo1), antioxidant, and/or detoxifying enzyme genes against oxidative stress (Gstm3, Gpx2) and cell signaling-related genes (Jun, Nfkbia) were investigated.

The mRNA levels of Cyp1a1 significantly increased in the Low/High I3C and PB+I3C groups when compared with the DEN-alone group, and levels in the PB+I3C group were significantly higher than the Low/High PB groups. The mRNA levels of Cyp2b1/2 and Cyp3a1 significantly increased in the PB- and/or I3C-treated groups when compared with the DEN-alone group, and these mRNA levels in the PB+I3C group were significantly higher than those in the DEN-alone and Low PB groups. In the PB+I3C group, the gene expression of Cyp2b1/2 was significantly lower than in the High I3C group. The mRNA levels of Nqo1 significantly increased in the High PB, Low/High I3C, and PB+I3C groups when compared with the DEN-alone group, and levels in the PB+I3C group were significantly higher than those in the Low/High PB groups (Table 3). In antioxidant and/or detoxifying enzyme genes against oxidative stress, the mRNA levels of Gstm3 and Gpx2 significantly increased in the PB- and/or I3C-treated groups when compared with the DEN-alone group, and the mRNA levels of Gstm3 in the DEN-alone and Low PB groups and Gpx2 in the DEN-alone and Low/High PB groups were significantly lower than in the PB+I3C group (Table 3). With regard to cell signaling-related genes, the mRNA levels of Jun significantly increased in the High I3C and PB+I3C groups when compared with the DEN-alone group, and levels in the PB+I3C group were significantly higher than in the DEN-alone and Low/High PB groups (Table 3).

When the results were analyzed by the two different additive statistical models, a suppressed induction of Cyp2b1/2 was observed in the PB+I3C group (the net value of the PB+I3C group, 20.85-fold; the sum of the net value of the low-dose groups of both PB and I3C, 36.01-fold; $p < 0.05$) in the heteroadditive model, and the same effect was detected in the PB+I3C group in the isoadditive model (the value of the PB+I3C group, 22.03 ± 2.30-fold; the average value of each individual treatment at a high dose of I3C or PB, 25.74 ± 3.88-fold; $p < 0.05$; Fig. 3B). In addition, there was an enhanced
induction of Nqo1 in the PB+I3C group (the value of the PB+I3C group, 16.41 ± 7.33-fold; the average value of that in each individual treatment at a high dose of I3C and PB, 7.17 ± 4.78-fold; p < 0.05) in the isoadditive model, but not in the heteroadditive model (the net value of the PB+I3C group, 15.40-fold; the sum of the net value of the low dose groups of both PB and I3C, 10.08-fold). There were no significant differences in the other genes in these models.

Microsomal ROS production
To estimate the cellular sources of ROS, NADPH-dependent ROS production derived from CYP activities in microsomal fractions was measured. As CYP’s monooxygenase activities need the reduction from NADPH, we compared ROS production with or without NADPH. Without NADPH, oxidized H$_2$DCFDA was not observed in any group. Conversely, with NADPH, the ROS production significantly increased in the PB- and/or I3C-treated groups when compared with the DEN-alone group, but the ROS production in the PB+I3C group was significantly higher than in the DEN-alone group only. The addition of SKF-525A, a CYP inhibitor, decreased ROS production in each group (Table 4). When the results were analyzed by the two different additive statistical models, a suppressed induction was observed in the PB+I3C group (the net value of the PB+I3C group, 61.77%; the sum of the net value of the low-dose groups of both PB and I3C, 101.12%; p < 0.05,) in the heteroadditive model, but not in the isoadditive model (the value of the combined administration of low doses of I3C and PB, 161.77 ± 28.42%; the average value of each individual treatment at a high dose of
To evaluate the oxidative damage of the cellular components caused by ROS production derived from CYP induction, hepatic TBARS formation was determined in the liver samples. TBARS levels significantly increased in the Low/High I3C and PB+I3C groups when compared with the DEN-alone group, and level in the PB+I3C group was significantly higher than the DEN-alone group (Table 4). There were no significant differences in TBARS level in the additive statistical model.

**DISCUSSION**

In the present study, we investigated the modifying effect of liver tumor promotion through co-administration of different types of CYP inducers, I3C (CYP1A1 inducer) and PB (CYP2B1 inducer), in rats. With regard to immunohistochemical results of GST-P, the co-administration of I3C and PB significantly increased the number and areas of GST-P+ foci compared with the DEN-alone group, and level in the PB+I3C group was significantly higher than the DEN-alone group (Table 4). There were no significant differences in TBARS level in the additive statistical model.

Therefore, when a significant fluctuation is observed in the isoadditve model rather than the heteroadditive model, it was regarded that such a fluctuation is biologically significant. Thus, the result of the present study suggests that the co-administration of I3C and PB causes no modifying effects in the liver tumor-promoting activity in rats. On the other hand, our previous studies demonstrated that the co-administration of CYP1A inducers, OPZ and BNF, at low doses induced increased numbers and area of GST-P+ foci when compared with the respective administration of OPZ and BNF at high doses and the administration of OPZ at high doses, respectively (Hayashi et al., 2012b). In addition, our group demonstrated that the co-administration of CYP2B inducers, PB and ORPH, at low doses significantly increased the numbers and area of GST-P+ foci compared with the respective administration of PB at low or high dose and of ORPH at a low dose, respectively (Morita et al., 2013). Therefore, there may be no modifying effect in terms of liver tumor promotion in rats subjected to combined administration of different types of CYP inducers, unlike the combined administration of the same type of CYP inducers.

The CYP family enzymes generate ROS as byproducts of microsomal oxidation, and the up-regulation of CYP1A1 and 1A2 isoforms indirectly results in the production of large amount of ROS (Pantarulo and Cederbaum, 1998; Nishikawa et al., 2002). It has been demonstrated that CYP1A1 inducers such as piperonyl butoxide, OX and BNF can generate ROS via a metabolic pathway in the liver and induce oxidative stress involved in hepatocarcinogenesis in rats (Dewa et al., 2008, 2009; Muguruma et al., 2007, 2009). Shimamoto et al. (2011b) suggested that I3C activates AhR and enhances microsomal ROS production following CYP1A induction, resulting in increased oxidative stress and potential liver damage.

**Table 4. Microsomal ROS production and TBARS levels in the livers of male F344 rats given PB and/or I3C for 6 weeks after DEN initiation**

<table>
<thead>
<tr>
<th>Group</th>
<th>DEN-alone</th>
<th>Low PB</th>
<th>High PB</th>
<th>Low I3C</th>
<th>High I3C</th>
<th>PB+I3C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of animals examined</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Microsomal ROS production (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>–NADPH</td>
<td>29.1 ± 2.3</td>
<td>26.3 ± 2.2</td>
<td>26.9 ± 3.0</td>
<td>20.2 ± 2.3</td>
<td>19.1 ± 1.9</td>
<td>20.0 ± 2.2</td>
</tr>
<tr>
<td>+NADPH</td>
<td>100.0 ± 13.5**</td>
<td>139.5 ± 15.6**</td>
<td>172.6 ± 22.2**</td>
<td>161.6 ± 14.8**</td>
<td>164.8 ± 11.8**</td>
<td>161.8 ± 28.4**</td>
</tr>
<tr>
<td>+NADPH+SKF-525A</td>
<td>38.1 ± 6.9</td>
<td>50.9 ± 7.9</td>
<td>58.6 ± 18.0</td>
<td>45.1 ± 7.4</td>
<td>48.2 ± 11.0</td>
<td>54.0 ± 9.8</td>
</tr>
<tr>
<td>TBARS level (nmol MDA/mg protein)</td>
<td>0.88 ± 0.06*</td>
<td>0.93 ± 0.05</td>
<td>0.97 ± 0.09</td>
<td>1.02 ± 0.08*</td>
<td>1.04 ± 0.05**</td>
<td>1.02 ± 0.13*</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± S.D.
* p < 0.05, ** p < 0.01 significantly different from the DEN-alone group (Dunnett’s test).
* p < 0.05, ** p < 0.01 significantly different from the PB+I3C group (Dunnett’s test).

I3C or PB, 168.66 ± 17.43%; Fig. 3C).

**Oxidative stress on cellular membrane**

To evaluate the oxidative damage of the cellular components caused by ROS production derived from CYP induction, hepatic TBARS formation was determined in the liver samples. TBARS levels significantly increased in the Low/High I3C and PB+I3C groups when compared with the DEN-alone group, and level in the PB+I3C group was significantly higher than the DEN-alone group (Table 4). There were no significant differences in TBARS level in the additive statistical model.
in the up-regulation of Nrf2 gene batteries, and such DNA oxidative stress subsequently induces liver tumor-promoting effects in rats. Similarly, PB, a CYP2B1/2 inducer, has been shown to increase hydroxyl radical levels in the livers of rats (Waxman and Azaroff, 1992; Kinoshita et al., 2003). Imaoka et al. (2004) demonstrated that CYP2B1 induction by PB induced ROS production and genomic DNA oxidation that may contribute to the liver tumor promotion by PB. In the present study, the mRNA expression levels of genes encoding phase I drug-metabolizing enzymes, Cyp1a1, Cyp2b1/2, Cyp3a1, and Nqo1 in the PB+I3C group were significantly higher than those in the Low PB group, but had no apparent change when compared with the Low I3C group. In addition, ROS production and TBARS levels had no apparent change among all treated groups. However, a suppressed induction of Cyp2b1/2 was observed in the PB+I3C group in the heteroadditive and isoadditive models. With regard to ROS production, a suppressed induction was observed in the PB+I3C group in the heteroadditive model, but not in the isoadditive model. Moreover, enhanced induction of Nqo1 was observed in the PB+I3C group in the isoadditive model, but not in the heteroadditive model. These results indicate that the co-administration of PB and I3C suppresses the induction of Cyp2B that can cause an inhibition of ROS generation. However, in the isoadditive model rather than the heteroadditive model, the ROS production was not suppressed in the PB+I3C group. Therefore, it was assumed that such a suppressed induction of Cyp2B was not enough to suppress the ROS production in this group. On the other hand, we could not clarify the reason why the co-administration of PB and I3C inhibited the induction of Cyp2B and enhanced the induction of Nqo1 that can eliminate the ROS generated in this group. We must clarify the possible mechanism of action on these fluctuations in a future study.

The mRNA levels of Gstm3 and Gpx2 in the PB+I3C group also had not changed compared with the Low PB or Low I3C groups. Gstm3 and Gpx2 are antioxidant genes that are regulated by Nrf2. Nrf2 is activated by oxidative stress and induces detoxification enzymes and antioxidants (Köhle and Bock, 2007). Under normal conditions, the Nrf2 protein is targeted as a ubiquitination site by Kelch-like ECH-associated protein 1 (Keap1), a member of the E3 ubiquitin ligase family in the cytoplasm. In contrast, under oxidative stress conditions, conformational changes in Keap1 make Nrf2 protein levels increase and the Nrf2 signaling pathway is activated (Kobayashi et al., 2006). Nrf2 gene batteries are involved in the protection against oxidative stress (Kwak et al., 2003). Glutathione S-transferases (GSTs) are a broadly expressed family of phase II isoenzymes that protect against endogenous oxidative stress (White et al., 2008). Gstm3, in the mu-class subfamily of GSTs, plays a pivotal role in conjugation and detoxification of environmental carcinogens (Hayes and Pulford, 1995). Gpx2 is a member of the glutathione peroxidase (GPX) family and reduces H2O2 (Naiki-Ito et al., 2007). The results of our study thus confirmed that expression levels of Nrf2 gene batteries were not enhanced by the combined administration of PB and I3C, suggesting that the ROS generation observed in the PB+I3C group was not so strong enough to induce the expression of Nrf2-related antioxidant enzyme genes in rats.

The ratio of Ki-67+ cells in the PB+I3C group at the outside of the GST-P+ foci was significantly higher than that in the Low PB and Low I3C groups, but there was no difference in Ki-67+ cell ratio in the PB+I3C group in the heteroadditive and isoadditive models. Thus, the negative findings in the additive statistical models indicate no enhanced cell proliferation at the outside and inside of the GST-P+ foci in the PB+I3C group. In addition, the mRNA levels of Jun in the PB+I3C group had not changed when compared with the Low PB and Low I3C groups, and no apparent difference in the mRNA levels of Nfkbia was observed in the experimental groups. Jun is one of the nuclear transcription factors related to carcinogenesis (Alcorn et al., 1990). The Nfkbia protein is the main regulator of NF-κB activation through conjugation with the NF-κB protein in cytoplasmic sequestration and inhibition of its transcriptional activation (Baumgarten and Frasor, 2012), and its expression was enhanced in carcinogenesis (Inoue et al., 2007). Therefore, these findings suggest that cell proliferation was not enhanced in the liver of rats given PB and I3C simultaneously, and support the no modifying effect in liver tumor promotion in this group.

In conclusion, we have demonstrated that combined administration of PB and I3C causes no modifying effects in liver tumor promotion in rats. However, the combined administration of two different CYP1A inducers causes a synergistic effect in liver tumor promoting in rats (Hayashi et al., 2012b), and the combined liver tumor-promoting effect of two different CYP2B inducers is synergistic in rats (Morita et al., 2013). Therefore, particular attention in risk assessments should be paid to the combined exposure of plural chemicals that are recognized as CYP inducers inducing the same CYP family. However, as in the results of our study, the concomitant exposure to the plural chemicals that have the capabilities to induce different CYPs does not always cause the enhanced liver tumor promotion. Given that our data are limited to the
combined administration of different CYP inducers, further examinations using other CYP inducers are vital.

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