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

Accession no.	Gene description	Symbol	Forward primer	Reverse primer
NM_013149	Aryl hydrocarbon receptor	*Ahr*	catcctggaaattcgaaccaa	gcatcacagccaataggtgtga
NM_010902	Nuclear factor, erythroid derived 2, like 2	*Nrf2*	tgcccctggaagtgtcaaa	ggctgtactgtatccccagaaga
NM_012540	Cytochrome P450, family 1, subfamily am polyoeptide 1	*Cyp1a1*	gccttcacatcagccacaga	ttgtgactctaaccacccagaatc
NM_012541	Cytochrome P450, family 1, subfamily am polyoeptide 2	*Cyp1a2*	aagcgccggtgcattg	tgcaggaggatggctaagaag
NM_022407	Aldehyde dehydrogenase 1 family, member A1	*Aldla1*	agtgccccttcggtggat	gctcagtgtactcataaagaccatgttc
NM_001039691	UDP glucuronosyltransferase 1 family, polypeptide A6	*Ugt1a6*	tggctaccccaaaacgatct	ataccatgggaaccggagtgt
NM_017000	NAD(P)H dehydrogenase, quinone 1	*Nqo1*	tccgccccaacttctg	tctgcgtgggccaataca
NM_183403	Glutathione peroxidase 2	*Gpx2*	accgatcccaagctcatcat	tctcaaagttccaggacacatctg
NM_001159739	Glutathione S-transferase Yc2 subunit	*Yc2*	aagctgagcagggctgatgt	acaatgcctgggtccatctc
NM_013215.	Aldo-keto reductase family 7, member A3	*Akr7a3*	ccgcttctttgggaatccat	ggcgatgccattgaagtgt
NM_012600	Malic enzyme 1, NADP(+)-dependent, cytosolic	*Me1*	cgaccagcaaagctgagtgtt	ctgccgctggcaaagatc
NM_053840	Gamma-glutamyltransferase 1	*Ggt1*	ccgcatcactgatgaaacca	cccatcgtctggaaggtagaat
NM_031144	Actin, beta	*Actb*	ccctggctcctagcaccat	agagccaccaatccacacaga

Lipid peroxidation levels

Oxidative lipid peroxidation was estimated using TBARS. Hepatic TBARS levels were determined using the method described by Ohkawa *et al.* (1979) with a slight modification. The liver tissue samples (approximately 50 mg) obtained from Experiment 2 were homogenized in 450 μl of buffer (containing 50 mM Tris-HCl; pH7.4, 1.15% KCl, 0.2 mM EDTA, 0.1 mM DTT, 0.1 mM Protease Inhibitor Cocktail and 20% glycerol) using TissueLyser (QIAGEN). Aliquots of 3.0 mg of liver homogenates were mixed with 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. Each reaction mixture was centrifuged at 4000 rpm for 10 min after adding 1.0 ml of distilled water and 5.0 ml n-butanol and pyridine (15:1, v/v). The absorbance of the resulting solution was determined spectrophotometrically at 532 nm, using a Synergy HT Multi-Detection Microplate Reader (BioTek, Winooski, VT, USA). The TBARS levels were expressed as the equivalents of malondialdehyde (MDA) amounts that were produced from 1,1,3,3,-tetramethoxypropane.

Preparation of microsomal fraction

The microsomal fractions were obtained according to the methods described by Yoshihara *et al.* (2001). The liver tissue samples (approximately 100 to 130 mg) obtained from Experiment 2 were homogenized in 700 μl of ice-cold buffer (containing 50 mM Tris-HCl; pH7.4, 1.15% KCl, 0.2 mM ethylene diamine tetra-acetic acid, 0.1 mM DTT, 0.1 mM Protease Inhibitor Cocktail and 20% glycerol) using a pestle. The homogenate was centrifuged at 700 × g for 10 min at 4°C, and supernatant was centrifuged at 10,000 × g for 20 min at 4°C. The 500 μl of supernatant was ultracentrifuged at 105,000 × g for 90 min. The microsomal pellet was resuspended in the microsome buffer, and protein content of the homogenate was measured using the BCA Protein Assay Kit (Pierce, IL, USA) with BSA as a standard.

Microsomal reactive oxygen species production

NADPH-dependent microsomal ROS production was determined by measuring the oxidation of 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) to its fluorescent product 2',7'-dichlorofluorescein (DCF) in liver microsomes according to the methods described by Schlezinger *et al.* (1999). The microsomes (final concentration 0.2 mg/ml) obtained from Experiment 2 were incubated in the dark at 37°C for 30 min in 50 mM Tris-HCl (pH 7.4) and 5 μM H_2DCFDA. In addition, 2.5 mM β-NADPH was added. In some cases, 0.1 mM SKF-525A (Toronto Research Chemicals, ON, Canada), a well-known inhibitor of cytochrome P450, and 0.1 mM H_2O_2 as a positive control were added to the wells. The fluorescence was monitored every 5 minutes over 2 hr using a Synergy HT Multi-Detection Microplate Reader (BioTek) with excitation and emission wavelengths of 485 and 528 nm, respectively. The data were then normalized to the control values, with the control expressed as a value of 100%.

Oxidative DNA damage levels

Oxidative DNA damage in the liver was estimated as 8-OHdG. The 8-OHdG levels in liver DNA was determined using the method of Umemura *et al.* (2006). Briefly, nuclear DNA obtained from Experiment 2 was isolated from 0.3 g of a wet weight sample, using a DNA Extractor WB Kit (Wako Pure Chemical Industries) containing an antioxidant NaI solution to dissolve the cellular components. For further prevention of autooxidation 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., Chelmstord, MA, USA) using previously reported running conditions (Umemura *et al.*, 2006).

Statistical analysis

All data were expressed as mean plus standard deviation. Numerical data were evaluated using the following methods: In Experiment 1, a Bartlett's test for equal variance was used to determine if the variance was homogenous between the groups. If the variance was homogenous, numerical data were assessed using Dunnett's multiple test. If a significant difference in variance was observed, Steel test was used instead. In Experiment 2, an F test for equal variance was used to determine if the variance was homogenous between the groups. If the variance was homogenous, numerical data were assessed using a t test. If a significant difference in variance was observed, the Welch's t test was used instead.

RESULTS

Experiment 1

Body and liver weights and food intake (Experiment 1)

In OPZ treated groups, the body weight was significantly lower than that of the DEN control group from 6 weeks after the start of experiment and the final body weight was significantly lower than that in the DEN control group (Table 2). Food intakes were not changed during the study period in the OPZ treated groups. The absolute and relative liver weights significantly increased in the OPZ treated groups compared to the DEN control group.

Histopathological examinations and GST-P positive foci in the liver (Experiment 1)

Histopathological analysis revealed centrilobular hypertrophy of hepatocytes, and foci of cellular alterations consisting of clear, eosinophilic and basophilic cells were induced in the OPZ treated groups given 138 and 276 mg/kg. Immunohistochemical analysis revealed that the number and area of GST-P positive foci significantly increased in the 276 mg/kg OPZ group compared with the DEN control group (Fig. 1, Table 2).

Experiment 2

Body and liver weights and food intake (Experiment 2)

In the DEN+ 276 mg/kg OPZ group, the body weight gain was lower than that of the DEN control group from 3 weeks after the start of experiment and the final body weight was significantly lower than the DEN control group (Table 3). Food intakes were not changed dur-

Table 2. Final body and liver weights, food intake and GST-P positive foci in the liver of male F344 rats treated with 0, 138 or 276 mg/kg OPZ for 6 weeks after DEN treatment (*Experiment 1*)

Group	DEN	DEN+OPZ 138 mg/kg	DEN+OPZ 276 mg/kg
No. of animals	10	11	12
Final body weight (g)	276.19 ± 11.4	258.9 ± 11.9**	255.6 ± 14.8**
Absolute liver weight (g)	8.36 ± 0.53	9.32 ± 0.67**	10.12 ± 0.84**
Relative liver weight (g/100 g body weight)	3.03 ± 0.24	3.60 ± 0.22**	3.98 ± 0.15**
Average food intake (g/kg body weight/day)	14.8 ± 1.1	14.5 ± 1.2	13.8 ± 1.1
GST-P positive foci (≥ 0.2 mm)			
Number (number/cm^2)	3.02 ± 1.91	4.47 ± 1.65	5.78 ± 2.18**
Area (mm^2/cm^2)	0.19 ± 0.12	0.37 ± 0.27	0.42 ± 0.20*

Data represent mean ± S.D.
*, ** Significantly different from DEN control at $p < 0.05$ or 0.01, respectively (Dunnett's multiple comparison test or Steel test).

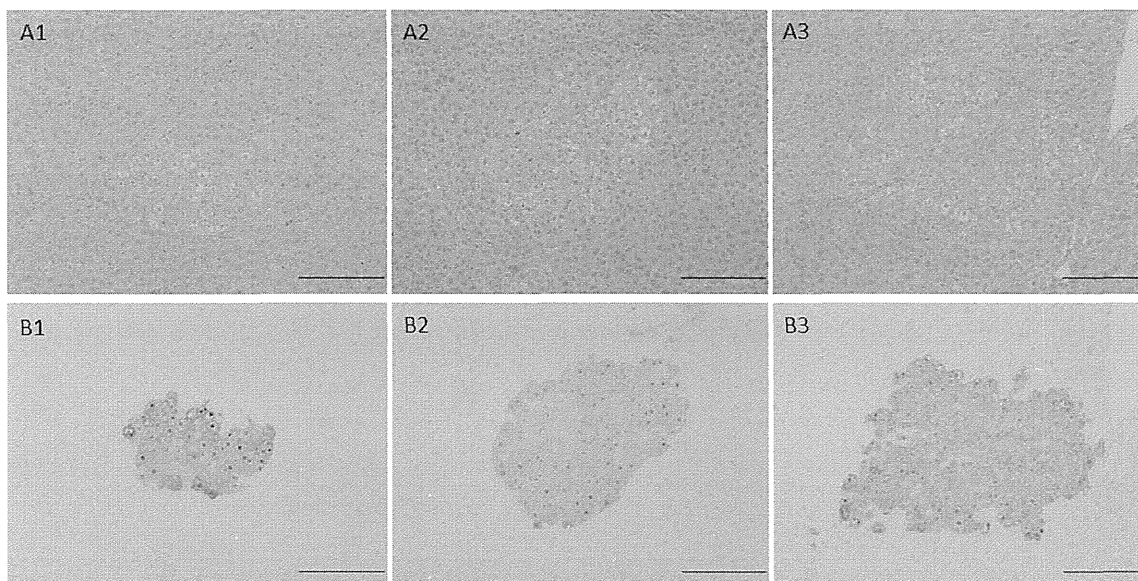

Fig. 1. Light microscopic and immunohistochemical photographs of hepatocellular altered focus. A1, 2 and 3: HE staining of the liver of partially hepatectomized rats treated with 0, 138 and 276 mg/kg OPZ after DEN initiation, respectively. B1, 2 and 3: GST-P staining in the livers of rats treated with 0, 138 and 276 mg/kg OPZ. Original magnification, respectively. × 100 (bar; 100 μm).

Table 3. Final total body and liver weights, average food intake, GST-P positive foci and PCNA positive hepatocytes in the liver of male F344 rats treated with 0 or 276 mg/kg OPZ for 6 weeks after DEN treatment (*Experiment 2*)

Group	DEN	DEN + 276 mg/kg OPZ
No. of animals	11	12
Final body weight (g)	274.2 ± 13.1	261.6 ± 16.8*
Absolute liver weight (g)	8.35 ± 0.46	10.52 ± 0.87**
Relative liver weight (g/100 g body weight)	3.06 ± 0.15	4.02 ± 0.18**
Average food intake (g/kg body weight/day)	14.0 ± 1.6	13.4 ± 2.0
GST-P positive foci (≧ 0.2 mm)		
Number (number/cm²)	2.65 ± 1.08	5.77 ± 2.46**
Area (mm²/cm²)	0.2 ± 0.14	0.41 ± 0.18**
PCNA-positive cells (%)	1.68 ± 0.87	3.8 ± 1.94**

Data represent mean ± S.D.
*, ** Significantly different from DEN control at $p < 0.05$ or 0.01, respectively (*t* test or Welch's *t* test).

ing the study period between the groups. The absolute and relative liver weights significantly increased in the DEN+OPZ group, being approximately 1.3-fold higher than those of the DEN control group.

Histopathological examinations and GST-P positive foci in the liver

Histopathological analysis revealed an increased number of foci of cellular alterations consisting of clear, eosinophilic and basophilic cells in the DEN+OPZ group.

Immunohistochemical analysis revealed that the number and area of GST-P-positive foci significantly increased in the DEN+OPZ group (Table 3). Moreover, the number of PCNA-positive cells also significantly increased in the DEN+OPZ group (Table 3).

DNA microarray and real-time RT-PCR analyses (Experiment 2)

Hepatic gene expression changes in rats treated with OPZ were screened using an oligonucleotide microarray. In the microarray, 177 or 251 genes showed more than a two-fold increase or more than a half-fold decrease, respectively, in their expression in one OPZ-treatment rat as compared with a rat of the DEN control group (supplementary data). Where gene expression was upregulated, we focused on the AhR battery and oxidative stress response-related genes. Real-time RT-PCR analysis on the genes listed in Table 1 was performed using liver samples (6 rats/group), except for the genes for *Cyp1a1* (11 rats/control group and 12 rats/OPZ group) (Table 4). In the AhR gene battery, a significant increase in the gene expression of *Cyp1a1*, *Cyp1a2*, *Nqo1*, *Ugt1a6* was observed in the DEN+OPZ group. In addition, antioxidant and/or detoxifying genes against oxidative stress such as *Gpx2*, *Yc2*, *Akr7a3*, *Aldh1a1*, *Me1* and *Ggt1* significantly increased in the DEN+OPZ group as compared with the DEN control group.

Microsomal ROS production (Experiment 2)

To estimate the cellular sources of ROS, NADPH-dependent ROS production was measured in liver microsomes (Table 5). The oxidized indicator was not detected in the absence of NADPH. By the addition of NADPH into the microsomal system, ROS production was dramatically enhanced, but ROS production in the DEN+OPZ group was less than that of the DEN control group. A well-known inhibitor of P450, SKF-525A, inhibited NADPH-dependent ROS production.

Oxidative stress on cellular membrane and DNA (Experiment 2)

To evaluate whether the oxidative damages to the cellular components occur, 8-OHdG content and TBARS formation were determined in the liver (Table 5). The content of 8-OHdG was not significantly changed in rats treated with OPZ as compared with that of the DEN control group. By contrast, TBARS content in the DEN+OPZ group was less than that of the DEN control group.

DISCUSSION

In a two-year carcinogenicity study of OPZ in rats, no

Table 4. Real-time RT-PCR analysis of liver tissues obtained from male F344 rats treated with 0 or 276 mg/kg OPZ for 6 weeks after DEN treatment (*Experiment 2*)

Gene symbol	DEN	DEN+OPZ 276 mg/kg
Ahr and Nrf2 transcripts		
Ahr	1.01 ± 0.13	2.62 ± 0.27**
Nrf2	1.03 ± 0.25	0.97 ± 0.25
Phase I drug metabolizing enzymes		
Cyp1a1	1.08 ± 0.49	1163.82 ± 1311.16**
Cyp1a2	1.00 ± 0.08	8.79 ± 4.10**
Ugt1a6	1.02 ± 0.2	2.59 ± 0.27**
Nqo1	1.01 ± 0.14	5.08 ± 1.55**
Phase II drug metabolizing enzymes		
Gpx2	1.05 ± 0.34	9.59 ± 3.54**
Yc2	1.09 ± 0.53	11.90 ± 3.48**
Akr7a3	1.09 ± 0.53	6.78 ± 3.01**
Aldh1a1	1.32 ± 0.29	4.17 ± 1.44**
Me1	1.01 ± 0.15	2.39 ± 0.53**
Ggt1	1.04 ± 0.16	3.23 ± 0.09**

Data represent mean ± S.D.
** Significantly different from DEN control at $p < 0.01$ (*t* test or Welch's *t* test).

Table 5. Microsomal ROS production, 8-OHdG and TBARS in the liver of male F344 rats treated with 0 or 276 mg/kg OPZ for 6 week after DEN treatment (*Experiment 2*)

Group	DEN	DEN + 276 mg/kg OPZ
No. of animals	6	6
ROS production (%)		
+NADPH	100 ± 15.7	57.3 ± 17.1**
+NADPH+SKF525A	7.8 ± 0.6	7.7 ± 0.7
-NADPH	47.3 ± 4.6	36.1 ± 6.2
8-OHdG (8-OHdG/10^5 dG)	0.62 ± 0.07	0.64 ± 0.16
TBARS (nmol MDA/mg protein)	0.93 ± 0.08	0.71 ± 0.04**

Data represent mean ± S.D.
** Significantly different from DEN control at $p < 0.01$ (*t* test or Welch's *t* test).

significant increase in the incidence of liver tumors was found. In contrast, in that study where female rats were treated orally with 138 mg/kg OPZ, it was found that this treatment regimen induced hepatocellular hyperplastic nodules in the liver (Ekman *et al.*, 1985). The term hepatocellular hyperplastic nodule is often used to describe non-neoplastic proliferative lesions in the liver of rodents which can be induced by repeated administration of certain chemicals causing degeneration and necrosis of hepatocytes (Maronpot *et al.*, 1986). In addition, a significant increase in the number of Ggt-positive foci was observed in the liver of rats subjected to oral administration of 100 mg/kg OPZ for 14 successive days after DEN treatment, further suggesting that OPZ treatment may confer greater susceptibility to tumorigenic changes (Mereto *et al.*, 1993). In Experiment 1 of the present study, the number and area of GST-P positive foci significantly increased in rats given 276 mg/kg OPZ after DEN treatment. To confirm the reproducibility of increased GST-P positive foci in OPZ treated groups of Experiment 1, we performed an additional experiment, Experiment 2. In Experiment 2, the number and area of GST-P positive foci also increased in the 276 mg/kg OPZ group. Additionally, the number of PCNA (a well-known cell proliferating marker) positive cells (Naryzhny, 2008) also increased in the DEN+OPZ group compared with the DEN control group. Since the medium-term liver carcinogenesis bioassay used in these studies has been repeatedly validated for the detection of hepatocarcinogens because of its high efficacy and very low false-positive rates (Ito *et al.*, 2003), these results from our study strongly indicate that OPZ has a liver tumor promoting effect in rats.

The genes *Cyp1a1/2*, *Nqo1* and *Ugt1a6* are well characterized as belonging to an 'AhR gene battery' (Köhle and Bock, 2007; Schrenk, 1998; Nebert *et al.*, 2000). In the present study, these genes significantly increased in the DEN+276 mg/kg OPZ group compared with the DEN control group, suggesting that OPZ activates AhR and induces upregulation of this AhR gene battery. Although CYP1A1 is expressed at low levels in extra-hepatic tissues in humans, it is significantly induced in the liver and extra-hepatic tissues by a range of chemicals (Ma and Lu, 2007). In comparison, CYP1A2 is constitutively expressed in the human liver, but is further induced by AhR agonists (Köhle and Bock, 2007). NQO1 detoxifies quinones by two-electron reduction to quinols without generating reactive semiqunines (Köhle and Bock, 2007) and UGT1A6 is a UDP-glycosyltransferase and conjugates a variety of planar phenols (Köhle and Bock, 2007). It is generally known that the cytochrome P-450 family generates ROS as byproducts of microsomal oxidation. CYP1A1 is a reactive CYP and generates a very large amount of ROS (Puntarulo and Cederbaum, 1998). It has been reported that the production of ROS due to increased CYP1A expression is induced not only by classical AhR ligands but also the atypical, non-AhR ligand such as OX (Dewa *et al.*, 2009). PCB also increases CYP1A1-dependent microsomal ROS production in the liver of rats (Schlezinger *et al.*, 2006). Therefore, upregulation of these genes suggest that ROS are generated in the microsome following treatment with OPZ.

Furthermore, the antioxidative genes regulated by Nrf2 such as *Gpx2*, *Yc2*, *Akr7a3*, *Aldh1a1*, *Me1* and *Ggt1* also significantly increased in the DEN+OPZ group. Nrf2, which is a transcriptional factor, regulates the expression of genes encoding the phase II detoxifying enzymes by binding to the antioxidant responsive elements (Itoh *et al.*, 1997), and is activated by oxidative/electrophile

stress (Köhle and Bock, 2007). GPX2 is a member of the glutathione peroxidase (GPX) family and reduces H_2O_2 (Naiki-Ito *et al.*, 2007). GSTs including *Yc2* detoxify xenobiotics by conjugating glutathione to a range of electrophilic substrates (Thimmulappa *et al.*, 2002). AKR7A3 is a NADPH-dependent oxido-reductase and catalyzes the reduction of aldehyde and keto groups (Thimmulappa *et al.*, 2002). ALDH1A1 has a substrate preference for the aldehyde products of lipid peroxidation such as malondialdehyde (Alnouti and Klaassen, 2008). ME1 is a NADPH generating enzyme, and increased induction of Me1 may be useful to the antioxidant effect (Thimmulappa *et al.*, 2002). GGT1 has an antioxidant effect and is involved in the metabolism of glutathione (Ravuri *et al.*, 2011). Upregulation of these genes suggests that the ROS produced following treatment of OPZ was probably eliminated as a result of the action of the genes found within this Nrf2 gene battery. In other words, OPZ induces microsomal ROS by the induction of AhR, and ROS production was probably attenuated by the induction of these xenobiotic metabolizing enzymes.

Since ROS production was predicted in the liver of rats given DEN+OPZ based on the upregulation of AhR and Nrf2 gene batteries, we therefore measured the oxidative stress markers including microsomal ROS, TBARS and 8-OHdG. However, microsomal ROS production and TBARS formation decreased in the DEN+276 mg/kg OPZ group compared with the DEN control group. In addition, 8-OHdG content remained unchanged in this group. These findings suggest that microsomal ROS generation, lipid peroxidation and DNA oxidative stress were not enhanced by the treatment of OPZ, in spite of the fact that the antioxidant genes were upregulated in the DEN+OPZ group. This means that the content of the ROS generation was not enough to induce lipid peroxidation and DNA oxidative stress. The plasma concentration of OPZ reached its peak (T_{max}) at 40.6 min, and then decreased to half levels after 24 min in male Sprague-Dawley rats given a single oral administration of OPZ (Lee *et al.*, 2007). Taking into account the rapid metabolism of OPZ, and given that the animals were subjected to oral OPZ dosing once a day in the present study, it can be speculated that ROS generation in the liver increases transiently, but most of the ROS produced are then eliminated by antioxidant enzymes. Therefore, the results obtained in the present study suggest that microsomal ROS is not directly involved in the liver tumor promotion mechanism of OPZ in rats, but we should clarify the more precise mechanism of the tumor promoting effect of OPZ in the future studies.

So far, we have demonstrated that several CYP1A inducers, including OX, BNF, piperonyl butoxide, and I3C, have liver tumor promoting activities in a two-stage hepatocarcinogenesis model in rats (Muguruma *et al.*, 2007; Shoda *et al.*, 2000; Dewa *et al.*, 2008; Shimamoto *et al.*, 2011b). These chemicals induce ROS generation in the microsomal fractions, and this ROS generation is suspected of enhancing liver tumor promotion by altering cellular physiological functions, such as oxidative proteins, lipid peroxidation, DNA damage and cell signaling (Muguruma *et al.*, 2007; Dewa *et al.*, 2008; Nishimura *et al.*, 2010; Shimamoto *et al.*, 2011a). Therefore, we speculated that most of the CYP1A inducers may have ROS-mediated liver tumor promoting activities in rats. However, we must reconsider our hypothesis, taking into account the finding of the present study that microsomal ROS is not involved in the liver tumor promotion of OPZ in rats.

Since OPZ is used for the treatment of dyspepsia, peptic ulcer, gastro-esophageal reflux disease, and the Zollinger-Ellison syndrome, we must pay an attention of the human risk of OPZ's tumor promoting effect. As the clinical dose recommended by the US Food and Drug Administraion (Howden, 1991; McTavish *et al.*, 1991), the therapeutic dose in human is 20 mg/day that is equivalent to 0.33 mg/kg body weight/day. On the other hand, in the present study, the DEN initiated rats were given 276 mg/kg OPZ. Therefore, this dose inducing the liver tumor promoting effect in rats is 828 times higher than the daily exposure dose of OPZ in human. This means that the human risk in the liver tumor promotion is extremely low when patients receive the OPZ's treatment.

In conclusion, we have confirmed that OPZ, CYP1A inducer, has a liver tumor promoting effect in rats, but the present study suggests that oxidative stress does not play a pivotal role in the liver tumor promotion of OPZ and further studies are necessary to clarify the precise mechanism of action.

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Original Article

Threshold dose of liver tumor promoting effect of β-naphthoflavone in rats

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ABSTRACT — To determine the threshold dose of β-Naphthoflavone (BNF) that induces hepatocellular tumor promoting effects, reactive oxygen species (ROS) generation and thiobarbituric acid-reactive substance (TBARS) formation, and drug-metabolizing enzymes that protect against ROS generation, two-stage liver carcinogenesis model was used. Partial hepatectomized rats (n = 11 to 12) were fed diets containing 0, 0.03, 0.06, 0.125 or 0.25% BNF for 6 weeks after an intraperitoneal injection of *N*-diethylnitrosamine (DEN) to initiate hepatocarcinogenesis. Histopathologically, glutathione *S*-transferase placental form (GST-P)-positive foci significantly increased in rats given 0.25% BNF. No marked changes in ROS production and TBARS contents were observed between the BNF treated and DEN alone groups. Real-time RT-PCR showed that the expression of *Cyp1a1*, *Cyp1a2*, *Cyp1b1* and *Nqo1* significantly increased in the groups given 0.03% BNF or more, but *Ugt1a6*, *Akr7a3* and *Gstm1* significantly increased in the groups given 0.125% BNF or more. *Gpx2* and *Yc2* significantly increased in the groups given 0.06% BNF or more and 0.25% BNF, respectively. Inflammation-related genes such as *Ccl2*, *Mmp12*, *Serpine1* and *Cox-2* significantly increased in the 0.25% BNF group. In immunohistochemistry, the number of cyclooxygenase-2 (COX-2)-positive cells increased in rats given 0.25% BNF. These results suggest that 0.25% BNF is the threshold dose for liver tumor promotion, and the fact that inflammation-related genes and COX-2 protein increased in the 0.25% BNF group strongly suggests that inflammation is involved in the liver tumor promoting effect of BNF in rats.

Key words: β-naphthoflavone, Threshold dose, Cyp1A inducer, Rat, Liver

INTRODUCTION

Oxidative stress has been recognized as an important facter affecting the pathogenesis of degenerative and inflammatory diseases, aging and cancer (Wiseman and Halliwell, 1996; Trush and Kensler, 1991). Especially, reactive oxygen Species (ROS) are considered to play an important role in the liver cancer, and ROS overproduction and subsequent oxidative DNA damage have been implicated to enhance the development of hepatocellular carcinomas that are caused by carcinogenic agents including hepatic C virus (Moriya *et al.*, 2001), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Lin *et al.*, 2007) and metals (Asare *et al.*, 2006). In addition, inflammation has also been recognized as a risk factor for carcinogenesis (Cervello and Montalto, 2006). Indeed, non-viral inflammation diseases of the liver including alcoholic hepatitis, hemochromatosis and primary biliary cirrhosis have been demonstrated to be related to the development of hepatocellular carcinomas (Cervello and Montalto, 2006).

β-Naphthoflavone (BNF) is a synthetic derivative of a naturally occurring flavonoid that selectively and strongly induces cytochrome P450 (CYP) 1A enzymes via aryl hydrocarbon receptor (AhR) activation (Guengerich and Liebler, 1985; Prochaska and Talalay, 1988). BNF does not have mutagenicity in Salmonella/microsome assay (Brown and Dietrich, 1979), but has a liver tumor promoting activity in a two-stage liver carcinogenesis bioassay in rats (Shoda *et al.*, 2000a, 2000b). In our previous study, the number and area of glutathione *S*-transferase placental form (GST-P)-positive foci significantly increased in the rat given diet containing 0.5 or 1% BNF for 6weeks after

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N-diethylnitrosamine (DEN) initiation treatment (Dewa *et al.*, 2008). In microarray and real-time RT-PCR analysis of the liver, AhR gene battery such as *Cyp1a1*, *Cyp1a2*, *Cyp1b1*, *Nqo1*, *Aldh3a1* and *Ugt1a6* as well as the transcriptional factor NF-E2-related factor2 (Nrf2)-regulated genes including *Gstm1*, *Gpx2*, *Akr7a3* and *Yc2* significantly increased in these BNF-treated rats compared to the DEN alone group. The enhancement of oxidative stress including microsomal ROS production, 8-hydroxydeoxyguanosine (8-OHdG), and thiobarbituric acid-reactive substance (TBARS) suggested that such an oxidative stress played an important role in the BNF-induced hepatocarcinogenesis in rats (Dewa *et al.*, 2008, 2009). In addition, our previous study demonstrated that inflammatory responses were also involved in the tumor promotion activity of BNF (Kuwata *et al.*, 2011). However, the threshold dose of the liver tumor promoting effect of BNF in rats has not been clarified.

In the present study, we have performed a two-stage liver carcinogenesis bioassay in rats to identify the threshold dose of BNF that induces liver tumor-promoting effects.

MATERIALS AND METHODS

Chemicals

BNF (CAS No. 6051-87-2, purity 98%) and DEN (CAS No. 55-18-5, purity 99%) were purchased from Wako Pure Chemical Industries (Osaka, Japan) and Tokyo Kasei Kogyo (Tokyo, Japan), respectively.

Animals and Experimental Design

Five-week-old male F344 rats were purchased from Japan SLC, Inc. (Shizuoka, Japan), maintained in an air-conditioned room with a twelve-hour light/dark cycle (room temperature, 23 ± 3°C; relative humidity, 55 ± 15%), and given free access to a basal diet (Oriental MF; Oriental Yeast, Tokyo, Japan) and tap water. The animals received human care in accordance with the Guide for Animal Experimentation of the Tokyo University of Agriculture and Technology.

After a one-week acclimatization period, a medium-term liver carcinogenesis bioassay (Ito *et al.*, 2003) was performed according to the following procedure: First, all rats were treated with an intraperitoneal injection of DEN at a dose of 200 mg/kg body weight, followed by treatment with 0 (DEN alone), 0.03, 0.06, 0.125 and 0.25% BNF in diet for six weeks starting two weeks after DEN treatment. To enhance hepatocellular proliferation, the rats were subjected to two-third partial hepatectomy in the first 3 weeks of commencing the experiment. The rational for dosage was determined based on the result of our previous study in which 0.5% BNF for 6 weeks showed tumor promoting effects in the liver of rats (Dewa *et al.*, 2008). At the end of the experiment, the rats were euthanized by exsanguination under ether anesthesia, and their livers were excised and weighed. The sliced liver samples were fixed in either 10% phosphate-buffered formalin for histopathological and immunohistochemical evaluations or were frozen in liquid nitrogen and stored at -80°C until further analysis.

Histopathology and immunohistochemistry

After formalin fixation, the tissues were dehydrated in graded ethanol and embedded in paraffin. Sections were then mounted onto the glass slides and stained with hematoxylin and eosin (H&E) or they were used for immunohistochemistry analysis. For immunohistochemistry, the horseradish peroxidase avidin-biotin complex method with a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) was used. Endogenous peroxidase was inhibited by incubation with freshly prepared 0.3% hydrogen peroxide with methanol for 30 min. The sections were incubated overnight with rabbit polyclonal anti-GST-P antibody (Medical & Biological Laboratories, Nagoya, Japan; 1:1,000), mouse monoclonal anti-proliferative cell nuclear antigen (PCNA) antibody (DAKO, Glostrup, Denmark; 1:800) and mouse monoclonal anti-cyclooxygenase (COX-2) antibody (BD Biosciences, Tokyo, Japan; 1:250) at 4°C, followed by incubation with a biotinylated secondary antibody for 30 min and with avidin peroxidase conjugate for 30 min at room temperature. The sections were then developed in 0.05% 3, 3'-diaminobenzidine/hydrogen peroxide as the chromogen. For PCNA staining and COX-2 staining, the deparaffinized tissue sections were placed in an antigen-retrieval solution (0.01 M citrate buffer, pH 6.0) for 20 min in a hot bath at 60°C or autoclaved at 121°C for 10 min prior to immunohistochemical staining. After staining, the slides were lightly counterstained with hematoxylin. Immunohistochemical analysis of GST-P and PCNA was performed on the liver of each rat from the all groups, but COX-2 immunostaining was performed on the DEN alone and 0.25% BNF groups.

The numbers and areas of GST-P positive foci (≥ 0.2 mm in diameter) and total areas of the liver sections were quantified using WinRoof software (v5.7.2; Mitani Corp., Fukui, Japan). The number of PCNA positive cells counted under × 200 magnification was expressed as a percentage of total cells counted in 20 randomly selected fields (including altered foci).

Real-time RT-PCR analyses

Total RNA was extracted with an RNeasy Mini Kit (QIAGEN, Hilden, Germany), in accordance with the manufacturer's instructions. Reverse transcription was carried out with 2 μg RNA for cDNA synthesis using a Thermoscript RT-PCR System kit (Eppendorf Co. Ltd., Tokyo, Japan) in accordance with the manufacturer's protocol. Quantitative real-time RT-PCR with Power SYBR Green PCR Master Mix (Applied Biosystems Japan Ltd., Tokyo, Japan) was performed using a StepOnePlus™ Real-time PCR System (Applied Biosystems Japan Ltd.). The PCR primers (listed in Table 1) were designed using Primer Express software (Version 3.0; Applied Biosystems Japan Ltd.). The amount of target gene expression was normalized to an endogenous reference (actin, beta) and relative to control was obtained using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Lipid peroxidation levels

Oxidative lipid peroxidation was estimated using TBARS. Hepatic TBARS levels were determined using the method described by Ohkawa *et al.* (1979) with a slight modification. The liver tissue samples (approximately 50 mg) were homogenized in 450 μl of buffer (containing 50 mM Tris-HCl; pH7.4, 1.15% KCl, 0.2 mM EDTA, 0.1 mM DTT, 0.1 mM Protease Inhibitor Cocktail and 20% glycerol) using TissueLyser (QIAGEN). Aliquots of 3.0 mg of liver homogenates were mixed with 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. Each reaction mixture was centrifuged at 3,400 rpm for 10 min after adding 1.0 ml of distilled water and 5.0 ml n-butanol and pyridine (15:1, v/v). The absorbance of the resulting solution was determined spectrophotometrically at 532 nm, using a Synergy HT Multi-Detection Microplate Reader (BioTek, Winooski, VT, USA). The TBARS levels were expressed as the equivalents of malondialdehyde (MDA) amounts that were produced from 1,1,3,3,-tetramethoxypropane.

Preparation of microsomal fraction

The microsomal fractions were obtained according to the methods described by Yoshihara *et al.* (2001). The liver tissue samples (approximately 100 to 130 mg) were homogenized in 700 μl of ice-cold buffer (containing 50 mM Tris-HCl; pH7.4, 1.15% KCl, 0.2 mM ethylene diamine tetra-acetic acid, 0.1 mM DTT, 0.1 mM Protease Inhibitor Cocktail and 20% glycerol) using a pestle. The homogenate was centrifuged at 3,000 rpm for 10 min at 4°C, and supernatant was centrifuged at 10,000 rpm for 20 min at 4°C. The 500 μl of supernatant was ultracentrifuged at 51,000 rpm for 90 min. The microsomal pellet was resuspended in the microsome buffer, and protein content of the homogenate was measured using the BCA Protein Assay Kit (Pierce, IL, USA) with bovine serum albumin as a standard.

Microsomal reactive oxygen species production

NADPH-dependent microsomal ROS production was determined by measuring the oxidation of 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) to its fluorescent product 2',7'-dichlorofluorescein (DCF) in liver microsomes according to the methods described by Schlezinger *et al.* (1999). The microsomes (final concentration 0.2 mg/ml) were incubated in the dark at 37°C for 30 min in 50 mM Tris-HCl (pH 7.4) and 5 μM H_2DCFDA. In addition, 2.5 mM β-NADPH was added. In some cases, 0.1 mM SKF-525A (Toronto Research Chemicals, ON, Canada), a well-known inhibitor of cytochrome P450, and 0.1 mM H_2O_2 as a positive control were added to the wells. The fluorescence was monitored every 5 min over 2 hr using a Synergy HT Multi-Detection Microplate Reader (BioTek) with excitation and emission wavelengths of 485 and 528 nm, respectively. The data were then normalized to the control values, with the control expressed as a value of 100%.

Statistical analysis

All data were expressed as mean plus standard deviation. Numerical data were evaluated using the following methods: Bartlett's test for equal variance was used to determine if the variance was homogenous between the groups. If the variance was homogenous, numerical data were assessed using the Dunnett's multiple test. If a significant difference in variance was observed, the Steel test was used instead.

RESULTS

Body and liver weights, food intake and histopathology

In all BNF treated groups, no significant changes were observed in body weight and food intake as compared with the DEN alone group (Table 2).

The absolute and relative liver weights significantly increased in the BNF treated groups. The relative liver weights in the 0.25% BNF group was approximately 1.4 fold higher than those in the DEN alone group (Table 2).

Histopathologically, BNF induced diffuse hepatocellular hypertrophy with eosinophilic cytoplasm in the groups given 0.06% BNF or more. Foci of cellular alterations of

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

Accession no.	Gene description	Symbol	Forward primer	Reverse primer
NM_012540	Cytochrome P450, family 1, subfamily am polyoeptide 1	*Cyp1a1*	gccttcacatcagccacaga	ttgtgactctaaccacccagaatc
NM_012541	Cytochrome P450, family 1, subfamily am polyoeptide 2	*Cyp1a2*	aagcgccggtgcattg	tgcaggaggatggctaagaag
NM_012940	Cytochrome P450, family 1, subfamily b, polypeptide 1	*Cyp1b1*	cttggccattgatcggaaa	caaggcgagcgaagtacaaagt
NM_017000	NAD(P)H dehydrogenase, quinone 1	*Nqo1*	tccgcccccaacttctg	tctgcgtgggccaataca
NM_001039691	UDP glucuronosyltransferase 1 family, polypeptide A6	*Ugt1a6*	tggctaccccaaaacgatct	ataccatgggaaccggagtgt
NM_013215	Aldo-keto reductase family 7, member A3	*Akr7a3*	ccgcttctttgggaatccat	ggcgatgccattgaagtgt
NM_017014	Glutathione S-transferase, mu 1	*Gstm1*	gaacgttcgcggacttactca	acgtatctcttctcctcatagtttgaatct
NM_183403	Glutathione peroxidase 2	*Gpx2*	accgatcccaagctcatcat	tctcaaagttccaggacacatctg
NM_001159739	Glutathione S-transferase Yc2 subunit	*Yc2*	aagctgagcagggctgatgt	acaatgcctgggtccatctc
NM_031530	Chemokine (C-C motif) ligand 2	*Ccl2*	aaaccagccaactctcactgaag	gcgtgacagagacctgcatagt
NM_053304	Collagen, type I, alpha 1	*Col1a1*	ttcacctacagcacgcttgtg	ttggtggttttgtattcgatgact
NM_012589	Interleukin 6	*Il6*	cccaccaggaacgaaagtca	cttgcggagagaaacttcatagc
NM_053963	Matrix metallopeptidase 12	*Mmp12*	gcgaggctgacattacgatactt	taaggtaccacctttgccatca
NM_012620	Serpin peptidase inhibitor, clade E , member 1	*Serpine1*	tggctcagaacaacaagttcaac	ggcagttccaggatgtcgtact
NM_017232	Cyclooxgenase 2	*Cox-2*	ttcgacttttccaggatggaa	gagtgtctttgactgtgggaggat
NM_012675	Tumor necrosis factor	*Tnf*	acaaggctgccccgactat	ctcctggtatgaagtggcaaatc
NM_031131	Transforming growth factor, beta 2	*Tgfb2*	cctgctgtaccttcataccgtcta	aatctcgcctccagctcttg
XM_342346	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	*Nfkb1*	gaagtacagaggaaacgcaccagaag	ccgccgccgaaactg
NM_001105720	Rattus norvegicus nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	*Nfkbia*	gcctagccccgagcattc	aatgatctgtttccccaaatttca
NM_017059	Bcl2-associated X protein	*Bax*	tggagctgcagaggatgattg	agctgccacccggaaga
NM_016993	B-cell CLL/lymphoma 2	*Bcl2*	ggcatctgcacacctggat	gagacagccaggagaaatcaaac
NM_030989	Tumor protein p53	*Tp53*	catgagcgttgctctgatggt	gatttccttccacccggataa
NM_171991	Cyclin B1	*Ccnb1*	tgtcccacacggaagaatctct	ggccacggttcaccatga
NM_080782	Cyclin-dependent kinase inhibitor 1A	*Cdkn1a (p21)*	cagccacaggcaccatgtc	cagacgacggcatactttgct
NM_130812	Cyclin-dependent kinase inhibitor 2B	*Cdkn2b (p15)*	ccctcaccagacctgtgcat	caggcgtcacacacatcca
NM_012524	CCAAT/enhancer binding protein (C/EBP), alpha	*Cebpa*	ggcgggaacgcaacaa	tctccacgttgcgctgttt
NM_031144	Actin, beta	*Actb*	ccctggctcctagcaccat	agagccaccaatccacacaga

clear cells, basophilic cells and eosinophilic cells were observed in the DEN alone and BNF treated groups, but the incidence of eosinophilic cells in the groups given 0.06% BNF or more was clearly higher than the DEN alone group (Table 3).

GST-P-positive foci and PCNA positive cells in the liver

Immunohistochemical analysis revealed that the number of GST-P-positive foci significantly increased in the 0.06 and 0.25% BNF groups, and the area of GST-P-positive foci significantly increased in the 0.25% BNF group (Table 3, Figs. 1a and b). Meanwhile, the number of PCNA-positive cells was not significantly changed between the BNF treated and DEN alone groups (Table 3).

Real-time RT-PCR analyses

In real-time RT-PCR analysis, a significant dose-dependent increase in the expression of *Cyp1a1*, *Cyp1a2*, *Cyp1b1* and *Nqo1* was observed in the BNF treated groups. BNF also increased the expression of *Ugt1a6, Akr7a3 and Gstm1* in the groups given 0.125% BNF or more (Table 4). In addition, the expression of *Gpx2* and *Yc2* significantly increased in the groups given 0.06% BNF or more and 0.25% BNF, respectively.

In addition, Inflammation-related genes such as *Ccl2*, *Mmp12*, *Serpine1 and Cox-2* significantly increased in the 0.25% BNF group compared with the DEN alone group. The expressions of *Col1a1*, *Il6*, *Tnf*, *Tgfb2 and Nfkb1* showed an increasing tendency in the BNF treated groups compared with the DEN alone group, but these changes were not statistically significant. On the contrary, the expression of *Nfkbia* significantly decreased in the group given 0.06% BNF or more.

With regard to the apoptosis-related genes, the expression of *Bcl2* significantly increased in the groups given 0.125% BNF or more, but the expression of *Bax* was not changed.

In the cell proliferation/cell cycle related genes, the expression of *p21* significantly decreased in the groups given 0.125% BNF or more, but the expression of *p15* significantly increased in the 0.25% BNF group. The expression of *p53* showed a decreasing tendency in the BNF treated groups, but there was no significant difference in this gene between the DEN alone and BNF treated groups.

Microsomal ROS production

To estimate the cellular sources of ROS, NADPH-dependent ROS production was measured in liver microsomes (Table 3). The oxidized indicator was not detected in the absence of NADPH. By the addition of NADPH into the microsomal system, ROS production was dramatically enhanced, but no marked changes in ROS production were observed between the BNF treated and DEN alone groups. A well-known inhibitor of cytochrome P450, SKF-525A, inhibited NADPH-dependent ROS production.

Oxidative stress on cellular membrane

To evaluate whether the oxidative damages to the cellular components occur, TBARS formation was determined in the liver (Table 3). There was no remarkable change in TBARS content between the BNF treated and DEN alone groups.

Immunohistochemistry of COX-2 in the liver

COX-2-positive cells were observed in sinusoid in the liver. Since their size was relatively large, they were considered as Kupffer cells. In the DEN control group, COX-2-positive cells were observed in periportal area of the liver, while the number of COX-2-positive cells in the 0.25% BNF group increased compared with the DEN alone group (Figs. 2a and b). They were scattered throughout the liver of the BNF group and especially expressed strongly in the periphery of the foci.

DISCUSSION

Our previous study demonstrated that BNF has a liver tumor promoting activity in rats given 0.5% BNF or more (Dewa *et al.*, 2008), and oxidative stress such as overproduction of microsomal ROS, lipid peroxidation and DNA damage was involved in the liver tumor promoting effect of BNF. In addition, treatment of 1% BNF for 28 weeks to rats induced altered foci and hepatocellular adenomas, and the enhancement of cell proliferation and protection against oxidative stress were related to the BNF-induced hepatocarcinogenesis in rats (Dewa *et al.*, 2009). Furthermore, concurrent apoptosis and regeneration of hepatocytes associated with inflammatory responses including TNFα-signaling have been shown to contribute to the tumor promotion of BNF (Kuwata *et al.*, 2011). In the present study, 0.25% BNF significantly increased the area and number of GST-P-positive foci in the two-stage hepatocarcinogenesis model in rats, and we confirmed that 0.25% BNF also has a liver tumor promoting activity in rats.

In the real-time RT-PCR, the expression of phase I drug metabolizing enzymes such as *Cyp1a1*, *Cyp1a2*, *Cyp1b1* and *Nqo1* significantly increased in the groups given 0.03% BNF or more. The cytochrome P-450 family is generally known to generate ROS as byproducts of microsomal oxidation. CYP1A1 has been reported to be

Table 2. Final body and liver weights, food intake and compound intake of male F344 rats given BNF for 6 week after DEN treatment

Group	DEN alone	DEN+0.03%BNF	DEN+0.06%BNF	DEN+0.125%BNF	DEN+0.25%BNF
Number of animals	11	12	12	11	11
Final body weight (g)	274.19 ± 13.14	277.57 ± 13.43	280.83 ± 17.16	280.00 ± 10.92	270.77 ± 12.78
Absolute liver weight (g)	8.35 ± 0.46	9.13 ± 0.60**	10.38 ± 1.14**	11.14 ± 0.61**	11.79 ± 0.93**
Relative liver weight (g/100 g body weight)	3.06 ± 0.15	3.29 ± 0.12**	3.69 ± 0.20**	3.95 ± 0.13**	4.36 ± 0.27**
Food intake (g/day/rat)	13.96 ± 1.65	14.37 ± 1.91	14.52 ± 2.60	13.94 ± 2.13	13.75 ± 2.97
Compound intake (mg/kg/day)	-	20.1 ± 4.0	40.0 ± 7.0	80.8 ± 16.3	163.0 ± 36.3

DEN: *N*-diethylnitrosamine, BNF: β-naphthoflavone. The data represent mean ± S.D.
** significantly different from the DEN alone group at P < 0.01.

Table 3. Incidence of foci of cellular alteration, number/area of GST-P-positive foci, PCNA-positive cells, ROS production and TBARS in the liver of male F344 rats given BNF for 6 week after DEN treatment

Group	DEN alone	DEN+0.03%BNF	DEN+0.06%BNF	DEN+0.125%BNF	DEN+0.25%BNF
Number of animals	11	12	12	11	11
Foci of cellular alterations					
Clear cells	10/11 (91%)	10/12 (83%)	7/12 (58%)	10/11 (91%)	8/11 (73%)
Basophilic cells	7/11 (45%)	7/12 (58%)	6/12 (50%)	6/11 (55%)	6/11 (55%)
Eosinophilic cells	1/11 (9%)	1/12 (8%)	6/12 (50%)	5/11 (45%)	7/11 (64%)
GST-P-positive foci (≧ 0.2 mm)					
Numbers (number/cm²)	4.01 ± 1.39	4.77 ± 1.27	6.01 ± 1.78*	4.97 ± 2.62	8.96 ± 3.15**
Areas (mm²/cm²)	0.29 ± 0.13	0.34 ± 0.14	0.44 ± 0.18	0.50 ± 0.50	0.86 ± 0.47*
PCNA-positive cells (%)	0.24 ± 0.17	0.24 ± 0.21	0.19 ± 0.08	0.14 ± 0.10	0.17 ± 0.14
ROS production (%)					
+NADPH	100 ± 6.7	97.9 ± 13.4	103.2 ± 15.6	98.4 ± 12.6	93.8 ± 12.8
+NADPH+SKF525A	41.4 ± 3.8	43.6 ± 4.8	46.4 ± 4.2	44.1 ± 3.6	43.0 ± 7.1
-NADPH	9.4 ± 0.5	9.1 ± 0.4	9.1 ± 0.6	9.4 ± 0.5	9.0 ± 0.7
TBARS (nmol MDA/mg protein)	1.46 ± 0.13	1.43 ± 0.21	1.25 ± 0.26	1.44 ± 0.21	1.46 ± 0.20

DEN: *N*-diethylnitrosamine, BNF: β-naphthoflavone. The data represent mean ± S.D.
*, ** significantly different from the DEN alone group at P < 0.05 or P < 0.01, respectively.

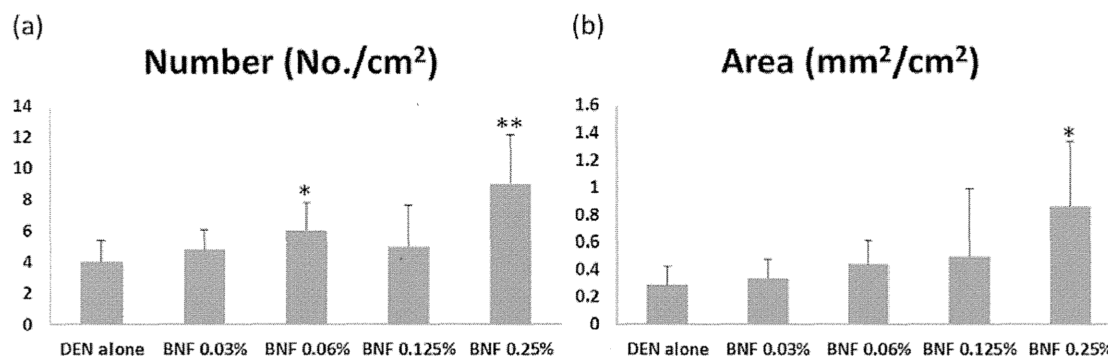

Fig. 1. The number and area of GST-P-positive foci in rats given BNF after DEN initiation. Each graph shows the area (a) and number (b) of GST-P positive foci in the liver of rats administered 0 (DEN alone), 0.03%, 0.06%, 0.125% and 0.25% BNF. Column represents mean + S.D. *, ** significantly different from the DEN alone group at $P < 0.05$ or $P < 0.01$, respectively.

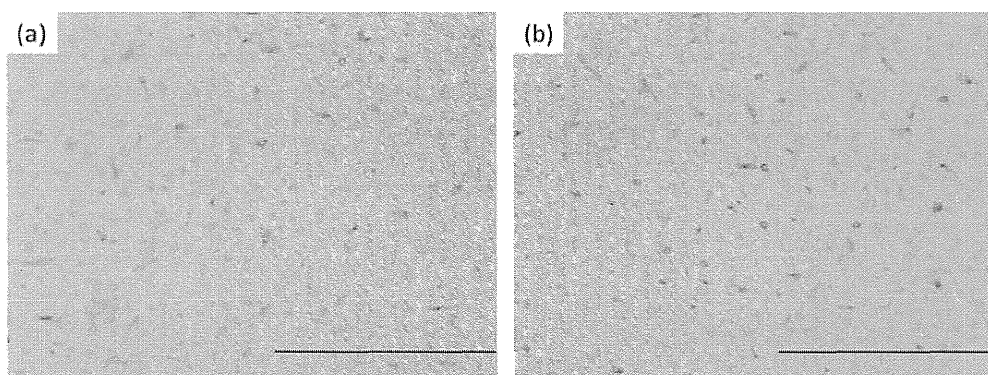

Fig. 2. Immunohistochemical photographs of COX-2 in the liver of rats given BNF after DEN initiation. Each photograph shows COX-2-positive cells in the liver of rats administered DEN alone (a) and 0.25% BNF (b). Original magnification × 200 (bar; 200 μm)

the major enzymes involved in the activation of most of the procarcinogens and promutagens (Guengerich and Shimada, 1991; Puntarulo and Cederbaum, 1998). The upregulation of CYP1A1 and 1A2 isoforms indirectly results in the production of a very large amount of ROS (Puntarulo and Cederbaum, 1998; Nishikawa *et al.*, 2002). Therefore, the upregulation of *Cyp1a1* by BNF treatment could have increased the amounts of ROS byproducts due to microsomal oxidation. On the other hand, the detoxification/antioxidant genes including *Akr7a3* and *Gstm1* significantly increased in the groups given 0.125% BNF or more, and *Gpx2* and *Yc2* significantly increased in the groups given 0.06% BNF or more and 0.25% BNF, respectively. These detoxification/antioxidant genes regulated by Nrf2, which is a transcriptional factor, are induced by the activation of Nrf2 by oxidative/electrophile stress (Köhle and Bock, 2007). Our previous study has shown that indole-3-carbinol (I3C) induced hepatocellular tumor promotion in parallel with the induction ratio of *Cyp1a1* and subsequent oxidative stress responses in rats (Shimamoto *et al.*, 2011). In the present study, the GST-P-positive foci increased in the 0.25% BNF together with the induction of *Cyp1a1* but not in the groups given 0.125% BNF or less despite of the high induction of *Cyp1a1*. These results suggest that 0.03% BNF or more induces ROS production due to the induction of *Cyp1a1* but 0.25% BNF only has a liver tumor promoting activity. However, microsomal ROS production and TBARS known as markers of oxidative stress were not changed in the BNF treated groups compared to the DEN alone group. Oxida-

Table 4. Real-time RT-PCR analysis of the liver tissues obtained from male F344 rats given BNF for 6 weeks after DEN treatment

Group	DEN alone	DEN+0.03%BNF	DEN+0.06%BNF	DEN+0.125%BNF	DEN+0.25%BNF
Phase I drug metabolizing enzymes					
Cyp1a1	1.04 ± 0.30	579.12 ± 216.81**	1922.87 ± 1055.00	5888.01 ± 994.34**	8670.44 ± 1637.65**
Cyp1a2	1.00 ± 0.10	3.24 ± 1.01**	7.78 ± 2.19*	16.35 ± 2.33**	29.42 ± 8.14*
Cyp1b1	1.09 ± 0.50	6.10 ± 1.39**	15.42 ± 4.86**	137.08 ± 56.92**	521.18 ± 210.40**
Nqo1	1.01 ± 0.14	2.35 ± 1.25**	3.03 ± 1.04**	5.57 ± 0.88**	9.47 ± 3.18**
Ugt1a6	1.03 ± 0.25	0.94 ± 1.78	0.96 ± 0.13	1.39 ± 0.18*	1.88 ± 0.34**
Phase II drug metabolizing enzymes					
Akr7a3	1.02 ± 0.21	1.37 ± 0.56	1.30 ± 0.50	2.45 ± 1.09*	3.24 ± 1.28**
Gstm1	1.04 ± 0.34	1.28 ± 0.50	1.54 ± 0.65	2.78 ± 1.21*	7.01 ± 2.47**
Gpx2	1.06 ± 0.40	1.57 ± 0.48	2.37 ± 0.89*	2.75 ± 0.43**	5.27 ± 1.22**
Yc2	1.02 ± 0.24	1.03 ± 0.30	0.93 ± 0.22	1.11 ± 0.27	1.43 ± 0.44*
Inflammation related genes					
Ccl2	1.11 ± 0.61	1.78 ± 0.67	2.09 ± 1.30	1.89 ± 0.47	2.18 ± 0.71*
Col1a1	1.28 ± 1.17	1.61 ± 1.57	1.51 ± 0.81	1.46 ± 0.42	2.16 ± 1.12
Il6	1.03 ± 0.25	1.68 ± 0.45*	2.12 ± 1.66	1.36 ± 0.34	2.15 ± 1.09
Mmp12	1.11 ± 0.59	0.99 ± 0.26	1.33 ± 0.66	1.35 ± 0.31	2.32 ± 1.13*
Serpine1	11.07 ± 0.47	1.24 ± 0.33	1.62 ± 0.50	1.76 ± 0.71	2.62 ± 1.46*
Cox-2	1.13 ± 0.59	1.86 ± 0.51	1.82 ± 1.04	2.02 ± 0.49	2.83 ± 0.97**
Tnf	1.04 ± 0.29	1.22 ± 0.22	1.44 ± 0.66	1.22 ± 0.24	1.55 ± 0.47
Tgfb2	1.05 ± 0.36	1.12 ± 0.43	0.97 ± 0.26	1.20 ± 0.21	1.45 ± 0.37
Nfkb1	1.01 ± 0.18	0.91 ± 0.13	1.02 ± 0.13	1.02 ± 0.16	1.14 ± 0.12
Nfkbia	1.01 ± 0.17	0.86 ± 0.16	0.74 ± 0.25*	0.64 ± 0.13**	0.67 ± 0.16**
Apoptosis related genes					
Bax	1.02 ± 0.24	1.12 ± 0.32	1.27 ± 0.58	0.90 ± 0.48	1.21 ± 0.29
Bcl-2	1.02 ± 0.21	1.24 ± 0.39	1.47 ± 0.28	1.70 ± 0.32**	2.36 ± 0.61**
Cell proliferation/Cell cycle related genes					
Tp53	1.02 ± 0.23	0.94 ± 0.23	0.82 ± 0.07	0.92 ± 0.20	0.89 ± 0.18
Ccnb1	1.02 ± 0.22	0.71 ± 0.19*	0.63 ± 0.19**	0.69 ± 0.14*	1.33 ± 0.27
Cdkn1a(p21)	1.03 ± 0.27	1.28 ± 0.43	0.76 ± 0.22	0.51 ± 0.11*	0.49 ± 0.26*
Cdkn2b(p15)	1.02 ± 0.25	1.23 ± 0.29	1.40 ± 0.54	1.33 ± 0.12	1.95 ± 0.38**
Cebpa	1.03 ± 0.28	1.00 ± 0.09	1.03 ± 0.19	0.87 ± 0.27	0.98 ± 0.20

DEN: *N*-diethylnitrosamine, BNF: β-naphthoflavone. The data represent mean ± S.D. Values are expressed as group mean fold change over control.
*, ** significantly different from the DEN alone group at $P < 0.05$ or $P < 0.01$, respectively.

tive stress occurs in cells or tissues when the amount of ROS produced exceeds antioxidant potential (Klaunig *et al.*, 1998). Therefore, it can be speculated that the antioxidant potential of these Nrf2-related enzymes exceeds the amount of ROS generation in the groups given 0.25% BNF or less and the ROS produced is accordingly eliminated from these enzymes.

In the present study, *Ccl2*, *Mmp12*, *Serpine1 and Cox-2* significantly increased in the 0.25% BNF group compared with the DEN alone group. These genes are known to be associated with inflammatory responses (Bertola *et al.*, 2010; Cobos-Correa *et al.*, 2009; Shimada *et al.*, 2010). *Tgfb2* induces *Serpine1* (Konrad *et al.*, 2009), and *Nfkb* and *Tnf* regulate the expression of *Ccl2* (Garg and Aggarwal, 2002). In addition, NFKB may regulate the production of prostaglandins (PG) via the COX-2 (Garg and Aggarwal, 2002). In the present study, COX-2-positive cells increased in the 0.25% BNF group compared with the DEN alone group. In the DEN alone group, COX-2-positive cells were observed in the periportal area of the liver. On the other hand, COX-2-positive cells were expressed throughout the liver in BNF treated groups and especially expressed strongly in the periphery of the foci. We thought that COX-2 was expressed in the Kupffer cells and BNF probably increased the number of Kupffer cells. COX-2 may be involved in the early stage of hepatocarcinogenesis, and increased expression of COX-2 in noncancerous liver tissues has been significantly associated with shorter disease-free survival in patients with hepatocellular carcinomas (Cervello and Montalto, 2006). In tumors, overexpression of COX-2 leads to an increase in PG levels, which affect many mechanisms involved in carcinogenesis such as angiogenesis, inhibition of apoptosis, stimulation of cell growth as well as the invasiveness and metastatic potential of tumor cells (Cervello and Montalto, 2006). In our previous study, enzymatically modified isoquercitrin (EMIQ) suppressed the liver tumor-promoting activity of BNF in rats through suppression of COX-2 (Shimada *et al.*, 2010). In addition, Kuwata *et al.* (2011) performed an additional mechanistic study on liver tumor promotion of BNF and clarified that BNF-induced oxidative stress cause single liver cell toxicity, allowing subsequent concomitant apoptosis and regeneration involving inflammatory responses including TNF-signaling, contributing to tumor promotion. Thus, inflammation is considered to be involved in the liver tumor promoting effect of BNF in rats.

Bcl2 is a proto-oncogene in blocking programed cell death rather than promoting proliferation, and a ratio of Bcl2/Bax determines the survival or death of cells following an apoptotic stimulus (Korsmeyer *et al.*, 1993). In addition, Bcl2 prevents oxidative damage to cellular constituents including cell membranes (Korsmeyer *et al.*, 1993). In the present study, the expression of *Bcl2* significantly increased in the groups given 0.125% BNF or more, but the expression of *Bax* was not changed in the BNF treated groups. Therefore, the exact mechanism of upregulated *Bcl2* is not clear, but *Bcl2* may be induced by ROS that was not completely eliminated from the antioxidant enzymes, and up-regulation of *Bcl2* probably leans the balance between cell survival and apoptosis to cell survival.

In cell proliferation activity, PCNA-positive cells were not significantly fluctuated in the BNF treated groups. P21 is an inhibitor of cyclin/cyclin-dependent kinase (CDK) complexes, and p21 induction is mediated by p53 (Roninson, 2002). Decreased expression of p21 protein contributed to the cell proliferation in altered foci and adenomas induced by BNF (Dewa *et al.*, 2009). In our study, the expression of *p21* decreased and the expression of *p53* slightly, but not significantly, decreased in the 0.25% BNF group. On the contrary, the expression of *p15*, one of CDK inhibitors which arrest cells in the G1 phase of the cell cycle, significantly increased in the 0.25% BNF group (Baldi *et al.*, 2011). Therefore, it can be considered that promotion and suppression of cell proliferation concurrently occurred in the highest dose group of BNF, and no marked cell proliferation is therefore induced in the BNF treated groups.

In conclusion, the result of our study showed that 0.25% BNF is the threshold dose for liver tumor promotion. The fact that oxidative stress was not induced in the doses of BNF used in this study while inflammation-related genes were upregulated and COX-2 protein were induced in the 0.25% BNF group strongly suggests that inflammation is involved in the liver tumor promoting effect of BNF in rats.

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Original Article

Enhanced liver tumor promotion but not liver initiation activity in rats subjected to combined administration of omeprazole and β-naphthoflavone

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ABSTRACT — Omeprazole (OPZ) and β-naphthoflavone (BNF) are cytochrome P450 (CYP)1A inducers and have liver tumor promoting effects. In this study, we investigated the co-promoting and co-initiating effects of OPZ and BNF in rats. In Experiment 1, male rats were subjected to partial hepatectomy (PH), and given oral doses of 138 or 276 mg/kg OPZ, 0.125% or 0.25% BNF or 138 mg/kg OPZ+0.125% BNF (n = 9~12) for 6 weeks after N-diethylnitrosamine (DEN) initiation. In Experiment 2, male rats were treated with oral doses of 138 or 276 mg/kg OPZ, 0.03% or 0.06% BNF or 138 mg/kg OPZ+0.03% BNF (n = 11~12) for 9 days starting 1 week before initiating treatment. As an initiating treatment, 2-Amino-3,4-dimethylimidazo[4,5-*f*]quinolone (MeIQx) was orally administered 12 hr after PH. The rats were fed a basal diet for 15 days, followed by a diet containing 0.015% 2-acetylaminofluorene for the next 10 days with a single oral dose of carbon tetrachloride. In Experiment 1, the number and area of glutathione *S*-transferase placental form-positive foci in the OPZ+BNF group were significantly higher than the average values of the High OPZ or the High BNF group. The expression of cyclooxygenase-2 (*Cox-2*) and COX-2 protein in the liver significantly increased in the OPZ+BNF group. In Experiment 2, liver initiation activity was not enhanced by the co-administration of OPZ+BNF. The results of our studies suggest that the co-administration of OPZ and BNF results in synergistic effects in the liver tumor promotion probably owing to increased COX-2 expression, but no modifying effect in the liver initiation activity of MeIQx in rats.

Key words: Omeprazole, β-naphthoflavone, CYP1A inducer, Liver tumor promotion, Rat

INTRODUCTION

Cytochrome P450 (CYP) 1A enzymes can be induced by a range of chemicals in a process mediated through the aryl hydrocarbon receptor (AhR) (Ma and Lu, 2007). Some CYP1A inducers such as 2,3,7,8-tetrachlorodibenzo-dioxin (TCDD) and β-naphthoflavone (BNF) are known to have liver tumor promoting effects in rats and the ability to augment oxidative stress (Kociba *et al.*, 1978; Dewa *et al.*, 2008). It is generally accepted that microsomal electron systems including CYPs and nicotinamide adenine dinucleotide phosphate (NADPH)-CYP reductase, generate reactive oxygen species (ROS) through metabolism, with the subsequent formation of an oxygenated substrate and water (Poulos and Raag, 1992). Although electron transfer is normally a well-coupled process, superoxide and H_2O_2 may be released in the presence of CYP1A inducers that are poorly metabolized.

We have previously demonstrated that some CYP1A inducers such as BNF, indole-3-carbinol and oxfendazole, have liver tumor promoting effects in rats and induce oxidative stress including microsomal ROS generation and thiobarbituric acid-reactive substances (TBARS) production in the liver (Dewa *et al.*, 2008, 2009; Shimamoto *et al.*, 2011a). We have also shown that a proton pump inhibitor, omeprazole (OPZ), which induces *Cyp1a*, also

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has a liver tumor promoting effect in rats, but oxidative stress is not involved in the mechanism (Hayashi *et al.*, 2012a).

More than 20 mutagenic and carcinogenic heterocyclic amines (HCAs) are produced by cooking or heating meat or fish (Hirata *et al.*, 2008). 2-Amino-3,4-dimethylimidazo[4,5-*f*]quinolone (MeIQx) is one of the major HCAs contained in cooked food consumed by humans (Tsuda *et al.*, 1999), and is thought to be a human liver carcinogen (Lynch *et al.*, 1992). MeIQx is metabolically activated to genotoxic intermediates by CYP1A2-mediated *N*-hydroxylation in the liver followed by *N*-acetyltransferase (NAT)-mediated *O*-esterification (Kato and Yamazoe, 1987; Yanagawa *et al.*, 1994). In our previous study, both OPZ and BNF have been shown to induce *Cyp1a2* expression in the liver of rats (Dewa *et al.*, 2008; Hayashi *et al.*, 2012b). Therefore, we speculated that simultaneous treatment of OPZ and BNF modifies MeIQx metabolism and enhances liver initiation activity in rats.

It has been demonstrated that simultaneous exposure to several chemicals at doses lower than the apparent carcinogenic doses results in marked carcinogenic actions in rats (Hasegawa *et al.*, 1989). In the present, regulatory agency in each country usually sets the acceptable daily intake and maximum residue limit for each chemical in food. However, if simultaneous exposures of plural chemicals enhance their adverse effects and/or initiation activities, we must reconsider the methods of risk evaluation. We think that the evaluation of the effect on combined administration of plural chemicals is beneficial for the risk assessment and risk management of chemicals contained in food. Therefore, we hypothesized that the combined administration of OPZ and BNF: (1) enhances more CYP1A induction resulting in more ROS generation and intensifies the liver tumor promotion effect; or (2) enhances more CYP1A2 induction resulting in more metabolic activation of MeIQx and intensifies the liver initiation effect.

In the present study, we investigated the modifying effect of liver tumor promotion and liver initiation through combined administration of OPZ and BNF in rats, with a particular focus on gene expression and the biochemical events of ROS generation and TBARS production in the liver.

MATERIALS AND METHODS

Chemicals

OPZ (CAS No. 73590-58-6, purity 98%), BNF (CAS No. 6051-87-2, purity 98%), MeIQx (CAS No. 77500-04-0, purity 99%) and carbon tetrachloride (CCl_4; CAS No. 56-23-5, purity 99.5%) were purchased from Wako Pure Chemical Industries (Osaka, Japan). *N*-Diethylnitrosamine (DEN; CAS No. 55-18-5, purity 99%) and 2-acetylaminofluorene (2-AAF; CAS No.53-96-3, purity 98%) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan).

Animals

Five-week-old male F344 rats used in Experiments 1 and 2 were purchased from Japan SLC Inc. (Shizuoka, Japan), maintained in an air-conditioned room with a 12-hr light/dark cycle (room temperature, 23°C ± 3°C; relative humidity, 55 ± 15%), and given free access to a basal diet (Oriental MF; Oriental Yeast, Tokyo, Japan) and tap water. After a 1-week acclimatization period, the animals were used in these experiments. The animals received humane care, in accordance with the Guide for Animal Experimentation of the Tokyo University of Agriculture and Technology.

Experimental designs

Experiment 1

A medium-term liver carcinogenesis bioassay (Ito *et al.*, 2003) was performed according to the following procedure (Fig. 1) to examine the modification of the liver tumor promoting effect by co-administration of OPZ and BNF. All rats were divided into 6 groups. First, all rats were given an intraperitoneal injection of DEN at a dose of 200 mg/kg body weight, followed by treatment with 0 (DEN alone), 138 mg/kg OPZ (Low OPZ) or 276 mg/kg OPZ (High OPZ) by oral gavage once a day, 0.125% BNF (Low BNF) or 0.25% BNF (High BNF) in diet, or 138 mg/kg OPZ + 0.125% BNF (OPZ+BNF) for 6 weeks starting 2 weeks after DEN treatment. Since the threshold tumor promotion dose of BNF and OPZ is 0.25% and 276 mg/kg, respectively (from our previous research), we adopted half of these doses as the low dose and combination dose in the present study (Hayashi *et al.*, 2012a, 2012b). To enhance hepatocellular proliferation, the rats were subjected to two-thirds partial hepatectomy (PH) at 1 week after the BNF/OPZ treatment was started. The food efficiency in each group was measured during the experiment. At the end of the experiment, the rats were euthanized by exsanguination under ether anesthesia and their livers were excised and weighed. The sliced liver samples were fixed in 10% phosphate-buffered formalin for histopathological and immunohistochemical examinations.