3.6. Immunohistochemical cellular distribution in the colon

In the colon, PhIP and CDCA did not induce a significant increase in any marker in the colon compared with the untreated controls (Fig. 6A–F). In comparison with the PhIP or CDCA group, no marker induced a significant change.

4. Discussion

In the present study, we found carcinogens that specifically targeted the thyroid, urinary bladder, forestomach and glandular stomach induced increases in Ki-67$^+$, nuclear Cdc2$^+$, p-Histone H3$^+$ and Aurora B$^+$ cells in their respective target organs. However, some carcinogens lacking induction of cell proliferation as estimated by the number of Ki-67$^+$ cells in the target organ, CDCA and PhIP in the colon did not increase all tested markers. We also previously found that carcinogens that evoked cell proliferation after 28 days of treatment increased apoptotic cells and cells expressing ubiquitin D (Ubd) at the G_2 phase, irrespective of target organs examined (Taniai et al., 2012). These results may suggest a sequential change of cell cycle aberration at the initial step of carcinogenesis facilitating target cell proliferation.

Experimentally, short-term treatment with carcinogens often induces proliferation of target cells (Akagi et al., 2003; Mally and Chipman, 2002). In the present study, SDM, PEITC, BHA and CC increased cell proliferation activity after 28 days of treatment. The hypothyroidism-related mechanism of SDM involves a decrease in the serum levels of T_3 and T_4, causing suppression of a negative feedback through the pituitary and an increase in the levels of serum thyroid-stimulating hormone (TSH) in rats (Imai et al., 2004). TSH then stimulates thyroid functions, including growth and proliferation of follicular cells as a link to carcinogenesis (Hard, 1998). PEITC induces cell proliferation of urinary epithelial cells after short-term administration because of cytotoxic oxidative DNA damage (Akagi et al., 2003). BHA induces cell proliferation of forestomach epithelium (Cantoreggi et al., 1993). CC induces cell proliferation in response to gastric epithelial erosion/ulceration (Hirose et al., 1999). In contrast, none of the carcinogens induced cell proliferation activity in non-target organs. These results suggest that there is a group of carcinogens, irrespective of target organs, that have the potential to induce proliferation of target cells in a 28-day treatment scheme. On the other hand, it has been reported that BHA enhanced cell proliferation in the esophagus, glandular stomach, small intestine and large intestine, in addition to the forestomach after oral treatment for 14 days in rats (Verhagen et al., 1990). However, BHA did not increase Ki-67$^+$ proliferating cells in the glandular stomach and colon in the present study. It has been previously reported that carcinogen-induced cell proliferation activity changes during the administration period (Akagi et al., 2003; Hirose et al., 1999). Therefore, it is possible that BHA no longer induces cell proliferation in the glandular stomach and colon after 28 days of treatment.

Cdc2 and cyclin B form the cyclin B–Cdc2 complex, which initiates the G_2/M transition, and nuclear localization of Cdc2 represents the active isoform entering at the M phase (Chan et al., 1999; Kawamoto et al., 1997). p-Histone H3 is crucial for chromosome condensation and segregation during mitosis (Nowak and Corces, 2004). Aurora B, a chromosomal passenger protein, plays a role in spindle assembly and chromosome segregation in mitosis (Meraldi et al., 2004). In the present study, SDM, PEITC, BHA and CC increased immunoreactive cells for nuclear Cdc2, p-Histone H3 and Aurora B, compared with the untreated controls, CL-treated controls and carcinogens targeting other organs. It has been demonstrated that prometaphase-arrested cells overexpress Cdc2 (Choi et al., 2011).

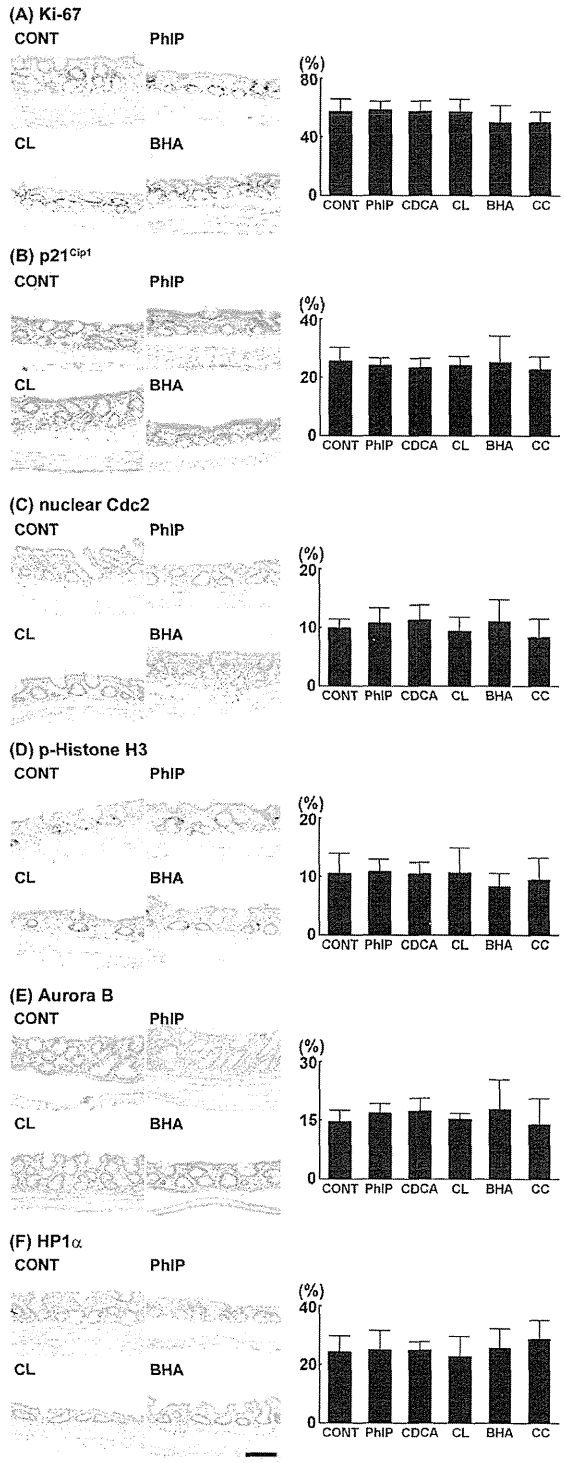

Fig. 6. Distribution of Ki-67$^+$, p21^{Cip1+}, nuclear Cdc2$^+$, p-Histone H3$^+$, Aurora B$^+$ and HP1α$^+$ cells in the colon. Photomicrographs show Ki-67$^+$, p21^{Cip1+}, nuclear Cdc2$^+$, p-Histone H3$^+$, Aurora B$^+$ and HP1α$^+$ cells in the colon in untreated controls and animals treated with PhIP, CL or BHA. The graphs show positive cell ratios (%) of epithelial cells per total cells counted in crypts using 10 animals per group. Values represent mean + SD. (A) Ki-67, (B) p21^{Cip1}, (C) nuclear Cdc2, (D) p-Histone H3, (E) Aurora B and (F) HP1α. Bar = 100 μm.

Overexpression of Aurora B causes chromosomal instability in various cancer cells (Qi et al., 2007) and increases in p-Histone H3 as a result of overexpression of Aurora B that contributes to chromosome number instability (Ota et al., 2002). These observations

suggest carcinogens that evoke cell proliferation increased cellular populations arrested at the M phase or showing chromosomal instability, irrespective of target organs. As we discussed previously, an aberrant increase of Ubd^+ cells at G_2 phase by carcinogens evoking cell proliferation in the target organ after a 28-day treatment may suggest an increase of the cellular population staying at the M phase (Taniai et al., 2012), because Ubd functions on the disruption of the spindle checkpoint at the M phase (Lim et al., 2006).

$p21^{Cip1}$ is one of the cyclin-dependent kinase (CDK) inhibitors that plays a role in G_1 checkpoint (Sherr and Roberts, 1995). Increased expression of this protein may link to G_1/S cell cycle arrest and apoptosis dependently with p53 (Liu et al., 2011). We previously found hepatocarcinogens that evoke liver cell proliferation evoked concomitant liver cell apoptosis that may link to p53 activation after 28-day treatment in rats (Yafune et al., 2013). In that study, we also found that all hepatocarcinogens tested increased $p21^{Cip1+}$ liver cells independently with cell proliferation activity (Yafune et al., 2013). Therefore, $p21^{Cip1}$ was expected to be an early prediction marker of carcinogens irrespective of the induction potential of target cell proliferation. However, in the present study, PEITC and BHA that evoked cell proliferation did not increase $p21^{Cip1+}$ cells in each target organ. It was previously reported that there was no obvious relationship between the expression of $p21^{Cip1}$ and cell proliferation activity in the development of proliferative lesions during carcinogenesis (Lu et al., 1999).

With regard to HP1α, only BHA induced an increase in immunoreactive cells in the forestomach epithelia, whereas SDM, PEITC and CC that induced cell proliferation did not increase $HP1\alpha^+$ cells in their target organ. It has been reported that HP1α plays a major role in chromosomal segregation during mitosis (Obuse et al., 2004), and phosphorylation of HP1α is necessary for genome stability (Hiragami-Hamada et al., 2011). Therefore, a phosphorylated cell population of HP1α rather than total cell population expressing HP1α may reflect a functional relationship of this protein for chromosomal maintenance.

We previously demonstrated that colon carcinogens (PhIP and CDCA) did not alter cellular distribution of proliferation markers, apoptosis index and G_2/M phase proteins (Taniai et al., 2012). In the present study, PhIP and CDCA also did not alter cellular distribution of the selected markers in colonic epithelia in accordance with the previous study. PhIP at 400 ppm in diet slightly induced colonic mucosal cell proliferation after 8-week administration, but the proliferation index was not increased after 4-week administration (Ochiai et al., 1996). It has been previously reported that CDCA administration for 2 weeks could induce cell proliferation of colonic epithelial cells after initiation with azoxymethane (Sutherland and Bird, 1994). However, CDCA without the initiation treatment only increased cell proliferation at low doses. CDCA at 1000 ppm in diet did not increase colonic mucosal cell proliferation in the present study. Therefore, it is possible that these colon carcinogens would not induce aberrant expression of M phase proteins in conjunction with activation of cell proliferation in a 28-day treatment scheme.

In conclusion, cell proliferation-evoking carcinogens in a 28-day treatment scheme can induce aberrant activation of M phase proteins reflecting cell cycle disruption, irrespective of target organs, which suggests an outcome of an increase in cell populations arrested at M phase or showing chromosomal instability. Carcinogens lacking proliferative activity did not have these effects. These results suggest that Ki-67 as a cell proliferation marker and nuclear Cdc2, p-Histone H3 and Aurora B as M phase proteins might functions as a rapid screening battery of biomarkers for carcinogens that exhibit high proliferative activity after a 28-day treatment in many target organs.

Conflict of interest statement

The authors disclose that there are no competing financial interests that could inappropriately influence the outcome of this study.

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Original Article

Liver tumor promoting effect of omeprazole in rats and its possible mechanism of action

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ABSTRACT — Omeprazole (OPZ), a proton pump inhibitor, is a cytochrome P450 (CYP) 1A1/2 inducer. Some CYP1A inducers are known to have liver tumor promoting effects in rats and the ability to enhance oxidative stress. In this study, we performed a two-stage liver carcinogenesis bioassay in rats to examine the tumor promoting effect of OPZ (Experiment 1) and to clarify a possible mechanism of action (Experiment 2). In Experiment 1, male F344 rats were subjected to a two-third partial hepatectomy, and treated with 0, 138 or 276 mg/kg OPZ by oral gavage once a day for six weeks after an intraperitoneal injection of *N*-diethylnitrosamine (DEN). Liver weights significantly increased in the DEN+OPZ groups, and the number and area of glutathione *S*-transferase placental form (GST-P) positive foci significantly increased in the DEN+276 mg/kg OPZ group. In Experiment 2, the same experiment as Experiment 1 was performed, but the dosage of OPZ was 0 or 276 mg/kg. The number and area of GST-P positive foci as well as liver weights significantly increased in the DEN+276 mg/kg OPZ group. The number of proliferative cell nuclear antigen (PCNA)-positive cells also significantly increased in the same group. Real-time RT-PCR showed that the expression of AhR battery genes including *Cyp1a1*, *Cyp1a2*, *Ugt1a6* and *Nqo1*, and Nrf2 battery genes including *Gpx2, Yc2, Akr7a3, Aldh1a1 Me1 and Ggt1* were significantly upregulated in this group. However, the production of microsomal reactive oxygen species (ROS) and formation of thiobarbituric acid-reactive substances (TBARS) decreased, and 8-hydroxydeoxyguanosine (8-OHdG) content remained unchanged in this group. These results indicate that OPZ, CYP1A inducer, is a liver tumor promoter in rats, but oxidative stress is not involved in the liver tumor promoting effect of OPZ.

Key words: Omeprazole, CYP1A1/2 inducer, Tumor promotion, Rat, Liver

INTRODUCTION

Omeprazole (OPZ) is a proton pump (H^+, K^+-ATPase) inhibitor characterized by a benzimidazole structure that is widely used as a blocker of gastric acid secretion in gastric parietal cells (Ma and Lu, 2007). As a result of its efficacy, OPZ is used clinically in humans for the treatment of dyspepsia, peptic ulcer, gastro-esophageal reflux disease, and the Zollinger-Ellison syndrome (Ma and Lu, 2007). In addition, it has been reported that OPZ has an anti-proliferation effect in pancreatic cancer cell line (Udelnow *et al.*, 2011). On the other hand, oncogenicity studies have found that OPZ may induce gastric carcinoids in rats, but not in mice (Ekman *et al.*, 1985), with the finding that hyperplasia of oxyntic mucosal cells (including hyperplasia of endocrine ECL-cells and development of gastric carcinoids in rats) can be attributed to the hypergastrinemia produced as a secondary effect of inhibition of acid secretion by the large doses of OPZ used (Ekaman *et al.*, 1985). It has also been reported that OPZ can induce cytochrome P450 (CYP) 1A1/2 expression in human hepatocytes *in vivo* and *in vitro* (Diaz *et al.*, 1990).

Both the CYP1A1 and CYP1A2 enzymes can be induced by a range of chemicals in a process mediated through the aryl hydrocarbon receptor (AhR) (Ma and Lu, 2007). AhR then heterodimerizes with the AhR nuclear translocator and the heterodimer binds to the cis-ele-

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ment called xenobiotic responsive element located in the promoter region of target genes to activate their transcription (Yoshinari *et al.*, 2008). The 'classical' AhR ligands are potent activators and include the halogenated aromatic hydrocarbons like 2,3,7,8-tetrachloro-dibenzo-dioxin (TCDD) and polyaromatic hydrocarbons such as 3-methylcholanthrene and polychlorinated biphenyls (PCBs), and β-naphthoflavone (BNF) (Coe *et al.*, 2006). In contrast, several compounds known as atypical CYP1A inducers induce CYP1A without direct binding to the AhR (Lemaire *et al*, 2004), including benzimidazole compounds such as oxfendazole (OX) and indole-3-carcinol (I3C) (Coe *et al.*, 2006; Gleizes-Escala *et al.*, 1996; Lesca *et al.*, 1995). Another benzimidazole compound, OPZ is also known to induce AhR; however, the molecular mechanism underlying this process is not yet clear.

Some CYP1A inducers such as TCDD and BNF are known to have liver tumor promoting effects in rats and the ability to augment oxidative stress (Kociba *et al.*, 1987; Dewa *et al.*, 2008). It is generally accepted that microsomal electron system including CYPs and NADPH-cytochrome P450 reductase generates reactive oxygen species (ROS) via 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. In contrast, atypical CYP1A inducers such as OX and I3C also have hepatocellular tumor promoting activities (Dewa *et al.*, 2009; Shimamoto *et al.*, 2011a), and promote hepatocellular tumors via oxidative stress. In long-term carcinogenicity studies of OPZ, hepatocarcinogenicity in rats given OPZ for 2 years was not found, but treatment with 138 mg/kg OPZ induced hepatocellular hyperplastic nodules in the liver of female rats (Ekman *et al.*, 1985). In addition, oral administration of 100 mg/kg OPZ for 14 successive days to rats which had previously been treated with *N*-diethylnitrosamine (DEN) led to a significant increase in the number of gamma-glutamyltranspeptidase (Ggt) positive foci in the liver (Mereto *et al.*, 1993).

In the present study, we have performed a two-stage liver carcinogenesis bioassay in rats to examine the tumor-promoting effects of OPZ in the liver and to clarify the possible mechanism of OPZ, with a particular focus on gene expression and biochemical events of ROS generation, 8-hydroxydeoxyguanosine (8-OHdG) and thiobarbituric acid-reactive substances (TBARS) production in the liver.

MATERIALS AND METHODS

Chemicals

OPZ (CAS No. 73590-58-6) and DEN (CAS No. 55-18-5) were purchased from Wako Pure Chemical Industries (Osaka, Japan) and Tokyo Kasei Kogyo (Tokyo, Japan) with purities of 98% and 99%, respectively.

Animals

Five-week-old male F344 rats in Experiments 1 and 2 were purchased from Japan SLC, Inc. (Shizuoka, Japan), maintained in an air-conditioned room with a twelve-hour 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 one-week acclimatization period, the animals were used in the experiments. The animals received human 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 to examine the liver tumor promoting effect of OPZ: 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), 138 or 276 mg/kg OPZ by oral gavage once a day for six weeks starting two weeks after DEN treatment. In the previous study, since 138 mg/kg OPZ induced hepatocellular hyperplastic nodules in the liver of female rats in a long-term carcinogenicity study (Ekman *et al.*, 1985), we adopted this dose as the low dose in the present study. In our previous pilot study (Data not shown), we conducted a 2-week repeated oral dose study of OPZ at doses of 0, 37.5, 75 or 150 mg/kg body weight/day in rats and confirmed that the expression of Phase I enzyme genes such as *Cyp1a1* inducing ROS generation and phase II enzyme genes to eliminate the ROS produced such as *Yc2*, *Gpx2*, *Akr7a3* and *Nqo1* were up-regulated in the rats treated with OPZ. The expression of these genes was up-regulated in a dose-dependent manner. Therefore, we did not set the untreated and OPZ alone groups in the present study. To enhance hepatocellular proliferation, the rats were subjected to two-third partial hepatectomy at 1 week after the OPZ treatment was started. In the first two weeks, the body weight was measured once a week, and then measured every three days during the promotion period, and the OPZ dose was then determined based on these data.

At the end of the experiment, the rats were euthanized by exsanguination while 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.

Experiment 2

The analysis of Experiment 1 revealed that 276 mg/kg OPZ had a liver tumor promoting activity. Therefore, we performed an additional experiment to clarify the possible mechanism of OPZ, with a particular focus on the oxidative stress. All rats were handled and treated in the same manner as in Experiment 1. In brief, two weeks after DEN treatment, the rats were administered 0 (DEN alone) or 276 mg/kg OPZ by oral gavage once a day for six weeks. In the first two weeks, the body weight was measured once a week, and then measured every three days during the promotion period, and the OPZ dose was then determined based on these data. At the end of the experiment, the rats were euthanized by exsanguination while 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 of livers in Experiments 1 and 2, 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-glutathione *S*-transferase placental form (GST-P) antibody (Medical & Biological Laboratories, Nagoya, Japan; 1:1000) and mouse monoclonal anti-proliferative cell nuclear antigen PCNA antibody (DAKO, Glostrup, Denmark; 1:800) 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, 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 prior to immunohistochemical staining. After staining, the slides were lightly counterstained with hematoxylin.

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. In both analyses, cranial and caudal parts of the right lateral liver lobe were used.

cDNA microarray analysis

Total RNA of the liver obtained from Experiment 2 was extracted with an RNeasy Mini Kit (QIAGEN, Hilden, Germany), in accordance with the manufacturer's instructions. Using 10 μg of total RNA from one animal from each of the control and 276 mg/kg OPZ groups, double-stranded cDNA was synthesized with the Invitrogen Superscript Double-Stranded cDNA Synthesis kit (Invitrogen Corp., Carlsbad, CA, USA), in accordance with the manufacturer's protocol. After labeling with the Cy3, 6 μg of each of the Cy3-labeled cDNA sample were loaded onto the *Rattus norvegicus* Roche NimbleGen microarray for Gene Expression (Roche NimbleGen: Euk Expr 385K catalog Arr, 26,739 targets/microarray). Using Robust Multiple Average normalization method (Irizarry *et al.*, 2003), differentially expressed genes were analyzed. Gene information was retrieved from the National Center for Biotechnology information (http://www.ncbi.nlm.nih.gov) website.

Real-time RT-PCR analysis

Total RNA of the liver obtained from Experiment 2 was extracted with an RNeasy Mini Kit (QIAGEN), 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).

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	*Aldh1a1*	agtgccccttcggtggat	gctcagtgtactcataaagaccatgttc
NM_001039691	UDP glucuronosyltransferase 1 family, polypeptide A6	*Ugt1a6*	tggctaccccaaaacgatct	ataccatgggaaccggagtgt
NM_017000	NAD(P)H dehydrogenase, quinone 1	*Nqo1*	tccgcccccaacttctg	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/cm²)	3.02 ± 1.91	4.47 ± 1.65	5.78 ± 2.18**
Area (mm²/cm²)	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).

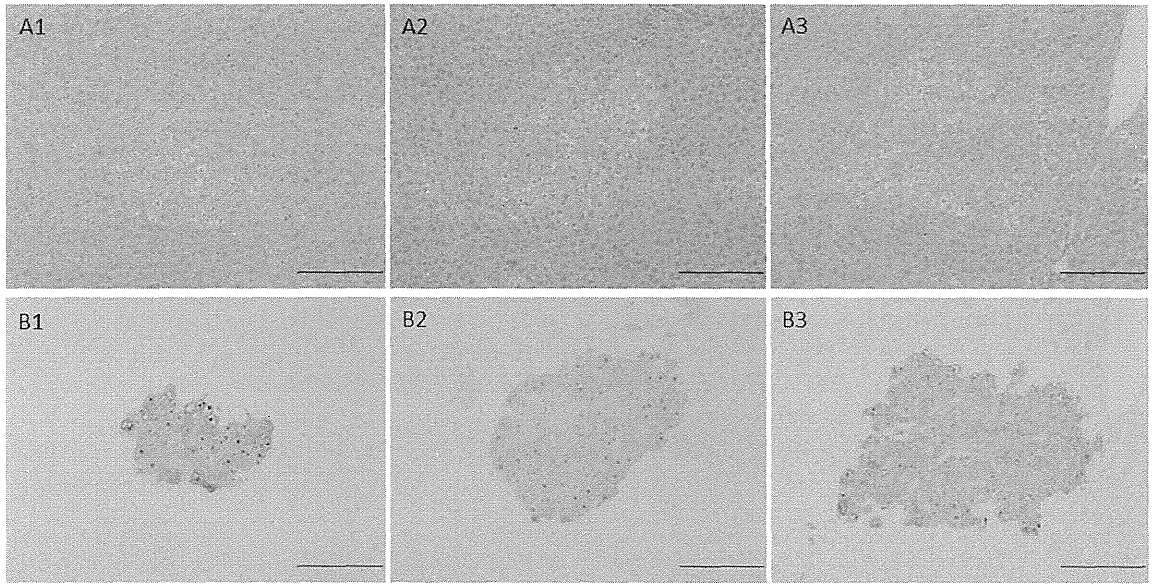

Fig. 1. Light microscopic and immunohistochemical photographs of hepatocellular altered focus. A1, 2 and 3: HE staining of the liver of partially hepatectomized rats treated with 0, 138 and 276 mg/kg OPZ after DEN initiation, respectively. B1, 2 and 3: GST-P staining in the livers of rats treated with 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). Up-regulation 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 ($\geq$ 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/cm^2)	4.01 ± 1.39	4.77 ± 1.27	6.01 ± 1.78*	4.97 ± 2.62	8.96 ± 3.15**
Areas (mm^2/cm^2)	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.