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 factor 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 inflam-

mation 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, $23 \pm 3^\circ\text{C}$; relative humidity, $55 \pm 15\%$), 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 ration-

al 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 $\times 200$ 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 μ g 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 μ l of buffer (containing 50 mM Tris-HCl; pH 7.4, 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 μ l of ice-cold buffer (containing 50 mM Tris-HCl; pH 7.4, 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 μ l 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 (H₂DCFDA) 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 μ M H₂DCFDA. 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 H₂O₂ 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 polypeptide 1	<i>Cyp1a1</i>	gccttcacatcagccacaga	ttgtgactctaaccaccaccagaatc
NM_012541	Cytochrome P450, family 1, subfamily am polypeptide 2	<i>Cyp1a2</i>	aagcgcgggtgcattg	tgcaaggagatggctaaagag
NM_012940	Cytochrome P450, family 1, subfamily b, polypeptide 1	<i>Cyp1b1</i>	cttggccattgatcggaaa	caaaggcagcgaagtaacaagt
NM_017000	NAD(P)H dehydrogenase, quinone 1	<i>Nqo1</i>	tccgcccccaactctg	tctgcgtgggccaataca
NM_001039691	UDP glucuronosyltransferase 1 family, polypeptide A6	<i>Ugt1a6</i>	tggtacccecaaaacgatct	ataccatgggaacccggagtg
NM_013215	Aldo-keto reductase family 7, member A3	<i>Akr7a3</i>	cgccttcttgggaatccat	ggcgcgatgcccattgaaagt
NM_017014	Glutathione S-transferase, mu 1	<i>Gstm1</i>	gaagcttgcggacttactca	acgtatctcttctcctatagtttgaatct
NM_183403	Glutathione peroxidase 2	<i>Gpx2</i>	accgatcccaagctcatcat	tctcaaaagtccaggagacacatctg
NM_001159739	Glutathione S-transferase Yc2 subunit	<i>Yc2</i>	aagctgagcagggctgatgt	acaatgcccgggtccatctc
NM_031530	Chemokine (C-C motif) ligand 2	<i>Ccl2</i>	aaaccagccaactctactgaag	ggctgacagagacctgcatagt
NM_053304	Collagen, type I, alpha 1	<i>Coll1a1</i>	ttcacctacagcagccttg	ttgggtgtttgttatcttgatgact
NM_012589	Interleukin 6	<i>Il6</i>	cccaccagggaacgaagica	cttgcggagagaacttcatagc
NM_053963	Matrix metalloproteinase 12	<i>Mmp12</i>	ggcggctgacattacgatactt	taaggtaaccctttggccatca
NM_012620	Serpin peptidase inhibitor, clade E, member 1	<i>Serpine1</i>	tggtctcagaacaacaagttcaac	ggcagttccaggatgctgact
NM_017232	Cyclooxygenase 2	<i>Cox-2</i>	ttcgactttccagggatggaa	gaggtgtctttgactgtgggagat
NM_012675	Tumor necrosis factor	<i>Tnf</i>	acaaggctgccccgactat	ctccctgggtatgaagtggcaaatc
NM_031131	Transforming growth factor, beta 2	<i>Tgfb2</i>	cctgctgtactctacacgicia	aatctcgccctccagctcttg
XM_342346	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	<i>Nfkb1</i>	ggaagtacagaggaaacgcaccagaag	cgcgcgcggaaactcg
NM_001105720	Rattus norvegicus nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	<i>Nfkbia</i>	ggcctagccccggagcattc	aatgatactgttttccccaaatttca
NM_017059	Bcl2-associated X protein	<i>Bax</i>	tggaagctgcagagggatgattg	agctggccaccgggaaga
NM_016993	B-cell CLL/lymphoma 2	<i>Bcl2</i>	ggcattctgcacacctggat	ggagacagccagggagaatcaaac
NM_030989	Tumor protein p53	<i>Trp53</i>	catgagcgttgcctctgatggt	gatttcttccaccgggataa
NM_171991	Cyclin B1	<i>Ccnb1</i>	tgctccacacggagaatctct	ggccacaggtttaccatga
NM_080782	Cyclin-dependent kinase inhibitor 1A	<i>Cdkn1a (p21)</i>	cagccacaggcacatgic	cagacgacggcatacttggct
NM_130812	Cyclin-dependent kinase inhibitor 2B	<i>Cdkn2b (p15)</i>	ccctcacacagacctgtgcat	caggcgttcacacacatcca
NM_012524	CCAAT/enhancer binding protein (C/EBP), alpha	<i>Cebpa</i>	ggcgggaacgcacaa	tctccactgtgcgtgttt
NM_031144	Actin, beta	<i>Actb</i>	ccctggctcttagccat	aggagcccaatccacacaga

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 *Colla1*, *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 *Nfkb1a* 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 TNF α -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 $\pm$ 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 ($\geq$ 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 $\pm$ S.D.
*, ** significantly different from the DEN alone group at $P < 0.05$ or $P < 0.01$, respectively.

Threshold dose of liver tumor promotion of β -naphthoflavone

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 $\times 200$ (bar; 200 μ m)

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					
<i>Cyp1a1</i>	1.04 ± 0.30	579.12 ± 216.81**	1922.87 ± 1055.00	5888.01 ± 994.34**	8670.44 ± 1637.65**
<i>Cyp1a2</i>	1.00 ± 0.10	3.24 ± 1.01**	7.78 ± 2.19*	16.35 ± 2.33**	29.42 ± 8.14*
<i>Cyp1b1</i>	1.09 ± 0.50	6.10 ± 1.39**	15.42 ± 4.86**	137.08 ± 56.92**	521.18 ± 210.40**
<i>Nqo1</i>	1.01 ± 0.14	2.35 ± 1.25**	3.03 ± 1.04**	5.57 ± 0.88**	9.47 ± 3.18**
<i>Ugt1a6</i>	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					
<i>Akr7a3</i>	1.02 ± 0.21	1.37 ± 0.56	1.30 ± 0.50	2.45 ± 1.09*	3.24 ± 1.28**
<i>Gstm1</i>	1.04 ± 0.34	1.28 ± 0.50	1.54 ± 0.65	2.78 ± 1.21*	7.01 ± 2.47**
<i>Gpx2</i>	1.06 ± 0.40	1.57 ± 0.48	2.37 ± 0.89*	2.75 ± 0.43**	5.27 ± 1.22**
<i>Yc2</i>	1.02 ± 0.24	1.03 ± 0.30	0.93 ± 0.22	1.11 ± 0.27	1.43 ± 0.44*
Inflammation related genes					
<i>Ccl2</i>	1.11 ± 0.61	1.78 ± 0.67	2.09 ± 1.30	1.89 ± 0.47	2.18 ± 0.71*
<i>Col1a1</i>	1.28 ± 1.17	1.61 ± 1.57	1.51 ± 0.81	1.46 ± 0.42	2.16 ± 1.12
<i>Il6</i>	1.03 ± 0.25	1.68 ± 0.45*	2.12 ± 1.66	1.36 ± 0.34	2.15 ± 1.09
<i>Mmp12</i>	1.11 ± 0.59	0.99 ± 0.26	1.33 ± 0.66	1.35 ± 0.31	2.32 ± 1.13*
<i>Serpine1</i>	11.07 ± 0.47	1.24 ± 0.33	1.62 ± 0.50	1.76 ± 0.71	2.62 ± 1.46*
<i>Cox-2</i>	1.13 ± 0.59	1.86 ± 0.51	1.82 ± 1.04	2.02 ± 0.49	2.83 ± 0.97**
<i>Tnf</i>	1.04 ± 0.29	1.22 ± 0.22	1.44 ± 0.66	1.22 ± 0.24	1.55 ± 0.47
<i>Tgfb2</i>	1.05 ± 0.36	1.12 ± 0.43	0.97 ± 0.26	1.20 ± 0.21	1.45 ± 0.37
<i>Nfkb1</i>	1.01 ± 0.18	0.91 ± 0.13	1.02 ± 0.13	1.02 ± 0.16	1.14 ± 0.12
<i>Nfkbia</i>	1.01 ± 0.17	0.86 ± 0.16	0.74 ± 0.25*	0.64 ± 0.13**	0.67 ± 0.16**
Apoptosis related genes					
<i>Bax</i>	1.02 ± 0.24	1.12 ± 0.32	1.27 ± 0.58	0.90 ± 0.48	1.21 ± 0.29
<i>Bcl-2</i>	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					
<i>Top53</i>	1.02 ± 0.23	0.94 ± 0.23	0.82 ± 0.07	0.92 ± 0.20	0.89 ± 0.18
<i>Ccnb1</i>	1.02 ± 0.22	0.71 ± 0.19*	0.63 ± 0.19**	0.69 ± 0.14*	1.33 ± 0.27
<i>Cdkn1a(p21)</i>	1.03 ± 0.27	1.28 ± 0.43	0.76 ± 0.22	0.51 ± 0.11*	0.49 ± 0.26*
<i>Cdkn2b(p15)</i>	1.02 ± 0.25	1.23 ± 0.29	1.40 ± 0.54	1.33 ± 0.12	1.95 ± 0.38**
<i>Cebpa</i>	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 $\pm$ 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, NF κ B 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 isquercitrin (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 programmed 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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