Table 1 List of primers used for real-time RT–PCR

Accession no.	Gene description	Gene symbol	Forward	Reverse
X00469	Cytochrome P450, family 1, subfamily a, polypeptide 1	*Cyp1a1*	gccttcacatcagccacaga	ttgtgactctaaccacccagaatc
NM_012541	Cytochrome P450, family 1, subfamily a, polypeptide 2	*Cyp1a2*	aagcgccggttgcattg	tgcaggaggatggctaagaag
XM_001070953	Cytochrome P450, family 2, subfamily b, polypeptide 2	*Cyp2b2*	gggacactgaaaaagagtgaagct	aatgccttcgccaagacaaat
NM_017000	NAD(P)H dehydrogenase, quinone 1	*Nqo1*	tccgcccccaacttctg	tctgcgtgggccaatataca
NM_020540	Glutathione S-transferase, mu 1	*Gstm1*	gaacgttcgcggacttactca	acgtatctcttctcctcatagtttgaatct
NM_183403	Glutathione peroxidase 2	*Gpx2*	accgatcccaagctcatca	tctcaaagttccaggacacatctg
NM_013215	Aldo–keto reductase family 7, member A3 (aflatoxin aldehyde reductase)	*Akr7a3*	ccgctttcttgggaatccat	ggcgatgccattgaagtgt
X78847	Glutathione S-transferase Yc2 subunit	*Yc2*	aagctgagcagggctgatgt	acaatgcctgggtccatctc
NM_012600	Malic enzyme 1	*Me1*	cgaccagcaaagctgagtgtt	ctgccgctggcaaagatc
NM_012583	Hypoxanthine guanine phosphoribosyl transferase	*Hprt*	gccgaccggttctgtcat	tcataacctggttcatcatcactaatc

Table 2 The effect of coadministration of EMIQ or MLT on body weights, food and water intake and liver weights of rats given OX after DEN initiation

Groups	DEN alone	DEN-OX	DEN-OX-EMIQ	DEN-OX-MLT	Untreated
Number of rats	9	10	11	12	5
Final body weight (g)	276.8 ± 21.2[a]	283.4 ± 23.3	258.6 ± 27.4[#]	255.1 ± 22.6[#]	303.2 ± 16.5
Food intake (g)[b]	16.9 ± 1.0	17.9 ± 1.4	17.3 ± 1.9	16.8 ± 2.2	17.8 ± 2.5
OX exposure (mg/kg BW/day)		38.2 ± 9.0	40.0 ± 9.0	38.7 ± 8.4	
Total OX intake (mg/head)		616.0	595.2	580.9	
Water intake (mL)[b]	19.1 ± 2.1	21.2 ± 2.3	21.3 ± 6.8	20.8 ± 3.6	15.2 ± 4.5
Antioxidant exposure (mg/kg BW/day)			201.2 ± 83.0	9.6 ± 2.4	
Total antioxidant intake (mg/head)			2,933.9	143.5	
Absolute liver weight (g)	6.94 ± 0.56	8.46 ± 0.97**	8.10 ± 1.00*	7.67 ± 0.80	8.12 ± 0.79
Relative liver weight (g)	2.51 ± 0.09	2.98 ± 0.10***	3.13 ± 0.18***,[#]	3.01 ± 0.13***	2.69 ± 0.38

[a] Values are expressed as mean ± SD

[b] Calculated from the weekly monitoring data

* $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$, significantly different from the DEN-alone group, as determined by Dunnett's rank or *d*-test

[#] $P < 0.05$, significantly different from the DEN-OX group, as determined by Dunnett's rank or *d*-test

was determined by the Dunnett's multiple comparison tests. A P value of less than 0.05 was considered statistically significant (Table 1).

Results

Although the final body weights in the DEN-OX-EMIQ and DEN-OX-MLT groups showed no significant changes when compared to those in the DEN-alone group, however, significant decreases were observed in the DEN-OX-EMIQ and DEN-OX-MLT groups when compared to those in the DEN-OX group (Table 2). No significant changes were observed in food and water intakes among the DEN-treated groups. With respect to the liver weight, absolute liver weights increased significantly in the DEN-OX and DEN-OX-EMIQ groups when compared to those in the DEN-alone group (Table 2). Relative liver weights increased significantly in all of the OX-treated groups when compared to those in the DEN-alone group. On the other hand, a significant increase in relative liver weights was observed in the DEN-OX-EMIQ group when compared to those in the DEN-OX group; however, this difference was considered to be due to the decreased final body weight of that group.

Histopathological examinations of livers from rats in the OX-treated groups indicated single cell necrosis in addition to hypertrophy and fatty degeneration of hepatocytes (Fig. 2). Hypertrophy and fatty degeneration of

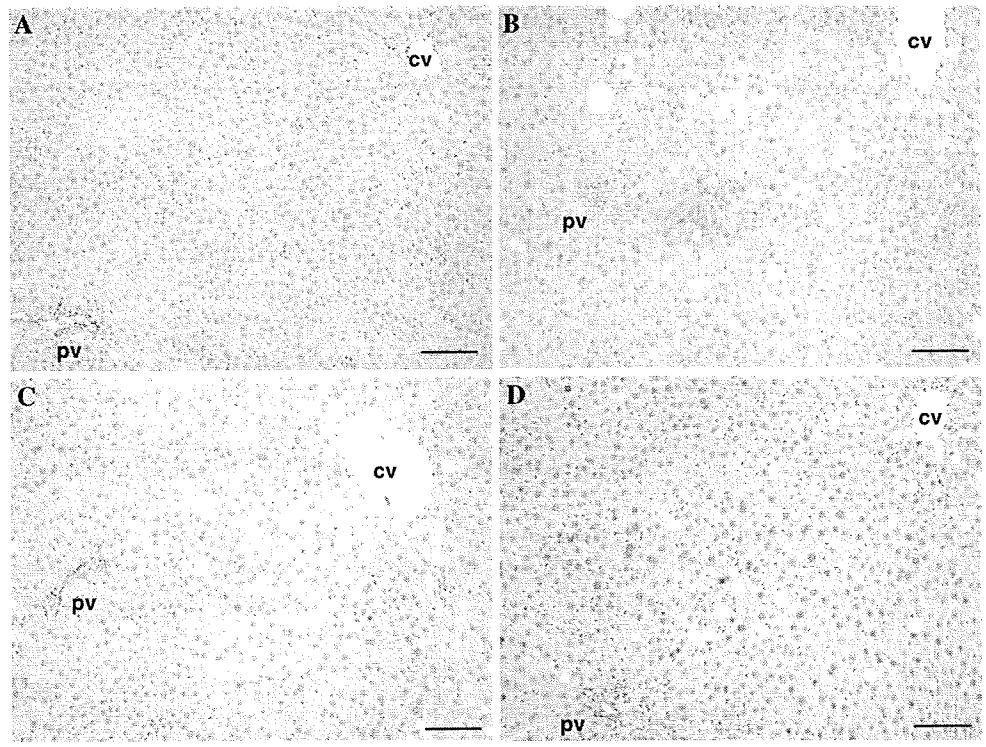

Fig. 2 Representative histopathological changes of H&E-stained liver sections of rats given **a** basal diet, **b** 500 ppm OX for 10 weeks, **c** 500 ppm OX and 2,000 ppm EMIQ for 10 weeks and **d** 500 ppm OX and 100 ppm MLT for 10 weeks. *Bar* = 100 μm. The abbreviations of "*cv*" and "*pv*" represent centrilobular vein and periportal vein, respectively. OX induces slight hypertrophy and vacuolation of hepatocytes

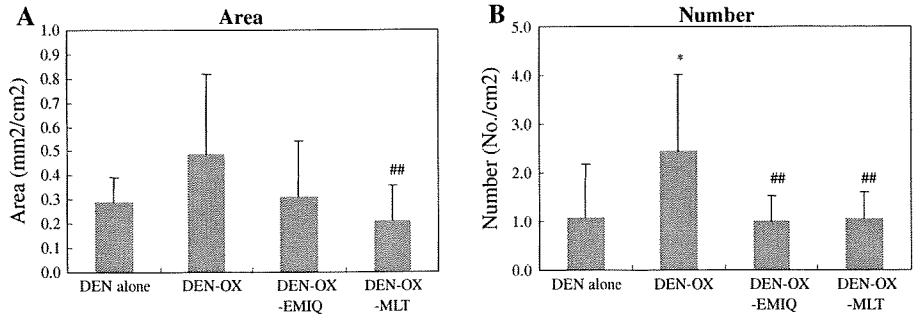

Fig. 3 The effect of coadministration of EMIQ or MLT on the area and number of GST-P-positive foci in the liver of rats given OX after DEN initiation. Values are expressed as mean ± SD of rats of DEN-alone group ($n = 9$), DEN-OX group ($n = 10$), DEN-OX-EMIQ group ($n = 11$) and DEN-OX-MLT group ($n = 12$). $^{*}P < 0.05$, significantly different from the DEN-alone group, as determined by Student's *t*-test or Aspin-welch *t*-test. $^{\#\#}P < 0.01$, significantly different from the DEN-OX group, as determined by Dunnett's rank or *d*-test

hepatocytes observed in rats given OX have been reported to be a compound-related effect in rats (WHO 1991). In addition, hepatocellular-altered foci were observed in the DEN-initiated groups, and GST-P-positive foci were observed in the DEN-OX groups. The area of GST-P-positive foci in the DEN-OX group showed a tendency of increase, and the number of GST-P-positive foci in the DEN-OX group significantly increased compared with that of the DEN-alone group. On the other hand, concomitant treatment with MLT suppressed the number and area of GST-P-positive foci compared to the DEN-OX group (Fig. 3). The number of GST-P-positive foci induced by OX was significantly inhibited by the administration of EMIQ.

In our previous study (Dewa et al. 2009), OX enhanced the oxidative stress which might contribute to its tumor-promoting potential in rats, and especially up-regulated not only mRNA expression of phase I enzymes *Cyp1a1* and *Cyp1a2*, but also Nrf2-regulated phase II enzymes such as *Gpx2*, *Nqo1*, *Yc2*, *Akr7a3* and *Gstm1* that were caused as an adaptive response against OX-induced oxidative stress. Therefore, in the present study, we also evaluated mRNA expression levels of *Cyp2b2* in addition to those genes in order to evaluate the effect of antioxidant EMIQ or MLT on the hepatic expression of xenobiotic detoxification- and oxidative stress-related genes in the livers of rats given OX. The results of real-time RT-PCR are shown in Table 3. As in the result of our previous study reported by Dewa et al.

Table 3 The effect of coadministration of EMIQ or MLT on mRNA expression levels in the livers of rats given OX after DEN initiation

Group	DEN alone	DEN + OX	DEN + OX + EMIQ	DEN + OX + MLT	Untreated
Gene name	5[a]	5	5	5	5
Cyp1a1	1.07 ± 0.46[b]	15.51 ± 2.95***	12.95 ± 3.74***	9.59 ± 2.72**,#	0.94 ± 0.55
Cyp1a2	1.00 ± 0.11	4.10 ± 0.67***	3.20 ± 0.52***	3.12 ± 0.85***	1.26 ± 0.20
Cyp2b2	1.03 ± 0.29	3.51 ± 0.70***	2.08 ± 0.18#	2.36 ± 0.84*,#	1.85 ± 0.58
Akr7a3	1.00 ± 0.08	2.59 ± 0.55***	2.03 ± 0.23**	1.68 ± 0.27*,#	1.20 ± 0.27
Me1	1.01 ± 0.16	2.50 ± 0.41***	1.79 ± 0.43*,#	1.65 ± 0.26*,##	1.19 ± 0.38
Nqo1	1.01 ± 0.17	2.53 ± 0.31*	2.66 ± 0.84**	2.30 ± 0.85*	0.63 ± 0.10
Gpx2	1.07 ± 0.48	3.51 ± 0.48**	3.31 ± 0.64**	4.42 ± 2.78**	0.71 ± 0.42
Gstm1	1.06 ± 0.38	4.74 ± 0.33***	6.18 ± 2.31***	4.73 ± 1.70***	0.93 ± 0.46
Yc2	1.06 ± 0.41	2.80 ± 1.08	3.10 ± 0.55*	3.95 ± 1.86*	0.55 ± 0.30

The mRNA expression levels are calculated according to the 2-ddCt method and normalized by *Hprt* as an endogenous control

[a] Number of rats examined

[b] Values of mRNA expression levels (normalized by *Hprt*) are expressed as mean ± SD

* $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$, significantly different from the DEN-alone group, as determined by Dunnett's rank or *d*-test

# $P < 0.05$ or ## $P < 0.01$, significantly different from the DEN-OX group, as determined by Dunnett's rank or *d*-test

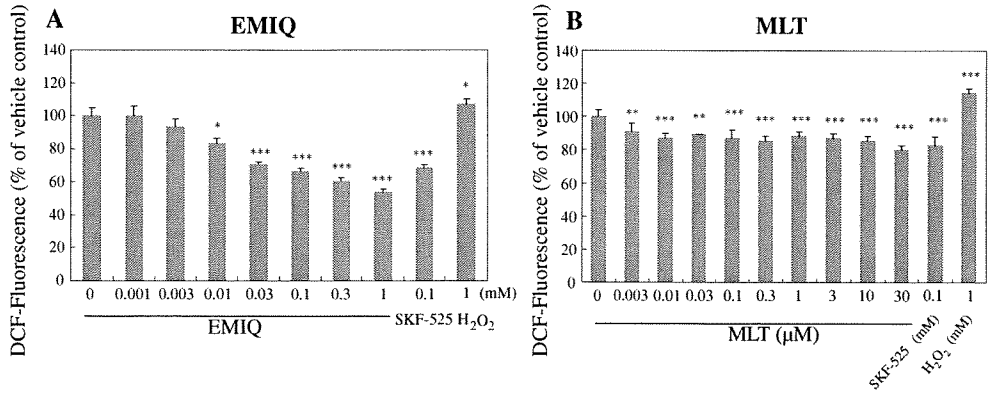

Fig. 4 The effect of the addition of EMIQ (**a**) or MLT (**b**) on hepatic microsomal ROS production of rats given OX after DEN initiation. The formation of DCF as a reactive marker of ROS was measured at an excitation of 485 nm/emission of 528 nm. *Columns* represent the relative value of fluorescent strength estimated at 100% in the absence of OX (vehicle control: 0 mM). The in vitro addition of SKF-525A or H_2O_2 significantly suppressed or increased ROS production in microsomes isolated from rats treated with OX, respectively. Values are expressed as mean ± SD. *$P < 0.05$, **$P < 0.01$ or ***$P < 0.001$, significantly different from the vehicle control values, as determined by Dunnett's rank or *d*-test

(2009), significant increases in the mRNA expression levels of *Cyp1a1*, *Cyp1a2*, *Akr7a3*, *Me1*, *Nqo1*, *Gpx2* and *Gstm1* in addition to *Cyp2b2* were observed in the DEN-OX group when compared with those in the DEN-alone group. On the other hand, the mRNA expression levels of *Cyp2b2* and *Me1* were significantly decreased in the DEN-OX-EMIQ group compared to those of the DEN-OX group. In the case of the DEN-OX-MLT group, decreased mRNA expression levels of *Cyp1a1* and *Akr7a3* were observed in addition to the decreased levels of *Cyp2b2* and *Me1* compared to those of the DEN-OX group.

The NADPH-dependent ROS production significantly decreased when treated with 10 μM of EMIQ or more or 0.003 μM of MLT or more (Fig. 4). Since the addition of SKF525A, which is a well-known inhibitor of P450, or H_2O_2 as positive control showed decrease or increase in ROS production, respectively, it was confirmed that the measuring system used in the assay of in vitro experiment was valid.

Discussion

In the present study, we demonstrated that the administration with EMIQ or MLT, which were reported to have an antioxidant potential (Zang et al. 1998; Cuzzocrea and Reiter 2001; Yokohira et al. 2008), significantly decreased the liver tumor-promoting activity of OX. These results indicate that EMIQ has an inhibitory effect on liver tumor promotion induced by OX. In addition, our results of the

present in vitro assay using hepatic microsomes strongly indicate that NADPH-dependent ROS production was decreased by the treatment with EMIQ or MLT. These results were compatible with the reports that MLT has activities related to the defense against ROS (Karbownik et al. 2000; Karbownik and Reiter 2000; Zang et al. 1998). Similarly, the fact that EMIQ has an inhibitory effect on ROS production indicates that the hepatocellular tumor promotion induced by OX is inhibited by the decrease in ROS production. These findings suggest that ROS production is involved in the hepatocellular tumor promotion of OX and strongly support our previous study (Dewa et al. 2009). Meanwhile, the minimum concentration (0.003 μM) of MLT in which a significant inhibition was observed in the in vitro assay was about ten times higher than the physiological concentrations (0.00003–0.0003 μM) of rats (Gerdin et al. 2004), but its concentration was considered to be within the range of fluctuations. On the other hand, with respect to EMIQ, details are unknown because of no information regarding plasma concentration, but Yokohira et al. (2008) have reported that rat serum after treatment with 1% EMIQ indicated significant high antioxidant power in vivo.

It has been reported that the hepatic microsomal ROS is produced in the liver of rats during the metabolic activation of different chemical carcinogens (Kadlubar and Hammons 1987). In particular, oxidative DNA damage, which may cause mutations and abnormal gene expressions, is considered to be one of the mechanisms of chemical carcinogenesis. Mitsumori et al. (1997) reported the increased expressions of CYP 1A1/2 and 2B1/2 protein in rats given OX. Dewa et al. (2009) also reported that OX has a potential for causing oxidative DNA damage and lipid peroxidation in the liver of rats, and its damage was partly derived from the enhancement in microsomal ROS production that is attributable to the increased mRNA expression of phase I enzyme, *Cyp1a1*/*Cyp1a2*, and protein expression of CYP1A1. Up-regulation of CYP1A1 and CYP1A2 isoforms has been reported to result indirectly in the production of very large amounts of ROS (Puntarulo and Cederbaum 1998; Canistro et al. 2002). Indeed, CYP1A1 and/or CYP1A2 induction in the liver was caused by chemicals such as TCDD (Park et al. 1996; Knerr et al. 2006), coplanar polychlorinated biphenyl congeners (Schlezinger et al. 2006), β-naphthoflavone (Dewa et al. 2008), dicyclanil (Moto et al. 2006) and piperonyl butoxide (Muguruma et al. 2007), which have hepatocellular tumor-promoting effects in addition to the generation of ROS. Similarly, increased hydroxyl radical levels were observed in rats given phenobarbital, which caused inductions of CYP2B1 and CYP2B2 (Waxman and Azaroff 1992; Kinoshita et al. 2002) and is known as a non-genotoxic liver tumor promoter (Peraino et al. 1971). In the present study, decreased mRNA expression levels of phase I enzyme *Cyp2b2* were observed in the case of coadministration with OX and EMIQ, and decreased mRNA expression levels of phase I enzyme *Cyp1a1* and *Cyp2b2* were found in the case of coadministration with OX and MLT. These results may indicate the decreases in total amounts of ROS produced by CYP enzymes by liver microsomes. However, since there is a possibility that other intracellular sources of ROS generation such as mitochondria (Shertzer et al. 2006), peroxisome (Schrader and Fahimi 2006) and Kupffer cells (Videla et al. 2003) might primarily contribute to cellular oxidative stress responses, further investigations are needed to clarify additional sources of ROS generation in the liver of rats treated with OX.

The mRNA expression levels of *Akr7a3*, which belong to the genes of phase II enzymes and are regulated under the transcriptional factor NF-E2-related factor 2 (Nrf2; Thimmulappa et al. 2002; Kwak et al. 2003; Dewa et al. 2007), and *Me1*, Nrf2-regulated gene (Thimmulappa et al. 2002) and phase II-related gene (Dewa et al. 2007) were observed in the present study. Me-1, which is a nicotinamide adenine dinucleotide phosphate (NADP)-dependent enzyme that generates NADPH, is known to be supplied for drug oxidation in hepatocytes (Thimmulappa et al. 2002) and has an ARE in its promoter region (Li et al. 2002). Aflatoxin B1 aldehyde reductase (AFAR), which was isolated and characterized from the livers of rats following administration of the antioxidant ethoxyquin (Judah et al. 1993), is known to have aflatoxin B1-detoxifying effects. AKR7A3, which belongs to the AFAR, is an enzyme characterized from an adult human liver cDNA library (Knight et al. 1999). As it has been reported that black tea polyphenols, which are known as one of the antioxidants, modulate the expression levels of phase I and phase II xenobiotic-metabolizing enzymes and oxidative stress in a rat hepatocarcinogenesis model using dimethylaminoazobenzene (DAB) and effectively suppress DAB-induced hepatocarcinogenesis (Murugan et al. 2008), these fluctuations were thought to be a possible cause of the inhibited hepatocarcinogenesis. Sulforaphane has a chemopreventive effect, which exerts its effect by strong induction of phase II enzymes via activation of Nrf2 (Chung et al. 2000; Ramos-Gomez et al. 2001; McMahon et al. 2001, Kwak et al. 2001). Thus, the induction of enzymes involved in their metabolism such as phase II enzymes is one of the important factors of protection against carcinogenesis, mutagenesis and toxicological changes. However, considering the decreased mRNA expressions of *Akr7a3* and *Me1* as well as the results of other genes observed in the present study, the mode of action against oxidative stress of MLT or EMIQ under the present experimental condition was considered to be different from that of sulforaphane. As the possibilities of modifying mechanism of these antioxidants, the following

four possibilities can be considered: (1) antioxidants may have a direct scavenging action of ROS, (2) antioxidants may have a decreasing effect on the expression of the CYP enzymes and generation of NADPH in vivo, (3) antioxidants may affect the pharmacokinetic profile of OX, and (4) antioxidants may affect the signaling pathways involved in the generation of ROS or be bound to any receptors involved in the growth of preneoplastic lesions. With respect to MLT, it has been reported that MLT molecules have high diffusion ability (Pieri et al. 1994; Reiter et al. 1999). These findings may lead to the fact that MLT is prone to have easier transmission to subcellular compartments, such as cytosol, nucleus, cellular membranes and mitochondria, and contributes to the direct scavenging action of ROS. In addition, it has been reported that MLT modulates the activity of several enzymes related to the antioxidative defense system (Pieri et al. 1994; Tan et al. 2000), such as glutathione, catalase and nitric oxide synthase (Montilla et al. 1997; Bettahi et al. 1996; Karbownik et al. 2001). Kimball et al. (2008) reported that the MLT has a modulating effect on the mitogen-activated protein kinase (MAPK) and mammalian target of rapamycin (mTOR) signaling pathways in H4IIE hepatoma cells. Li and Witt-Enderby (2000) reported that numerous physiological effects such as circadian rhythm regulation, sleep disturbances, seasonal reproduction, retinal physiology, immune function and intermediary metabolism by MLT involve inhibitory G protein-coupled melatonin receptors leading to the suppression of cAMP production. On the other hands, with respect to the possibility of drug interactions between OX and antioxidants, judging from the results obtained in the present study, the possibility that antioxidants affect the excretion ability of OX subjected to glutathione conjugation could be denied, because the modulating effect on the expression of *Gstm1* mRNA was not observed in the antioxidant-treated groups when compared with the DEN-OX group. As described earlier, various possibilities are considered, but the possible mechanism on inhibitory action of the onset of preneoplastic lesions induced by OX cannot be elucidated at the present time. In vivo data providing direct evidence that EMIQ and MLT were able to exert antioxidative action may lead to breakthrough of the mechanism. Further studies are necessary to clarify the possible mechanism on the inhibitory action.

In conclusion, we have demonstrated that ROS generated by the activation of CYPs contributes to the OX-induced liver tumor-promoting effect in rats. In addition, since EMIQ as well as MLT have an antioxidant potential and inhibit the hepatocellular tumor-promoting action of OX, the result of the present study may suggest that EMIQ has an activity of tumor inhibition and probably prevents the development of tumors.

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Original Article

The threshold dose for liver tumor promoting effects of dicyclanil in ICR mice

Meilan Jin[1,2], Yasuaki Dewa[1], Masaomi Kawai[1,3], Jihei Nishimura[1], Yukie Saegusa[1,3], Sayaka Kemmochi[1,3], Tomoaki Harada[1], Makoto Shibutani[1] and Kunitoshi Mitsumori[1]

[1]Laboratory of Veterinary Pathology, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu-shi, Tokyo 183-8509, Japan
[2]Division of Pathology, Biological Safety Research Center, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan
[3]Pathogenetic Veterinary Science, United Graduate School of Veterinary Sciences, Gifu University, 1-1 Yanagido, Gifu-shi, Gifu 501-1193, Japan

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ABSTRACT — To determine the threshold dose of dicyclanil (DC) that induces hepatocellular tumor-promoting effects associated with reactive oxygen species (ROS) generation via their metabolic pathways, partial hepatectomized ICR male mice were fed diets containing 0, 187.5, 375 or 750 ppm DC after an intraperitoneal injection of N-diethylnitrosamine (DEN) to initiate hepatocarcinogenesis. Immunohistochemically, the proliferating cell nuclear antigen (PCNA)-positive cell ratio was significantly increased in the DEN + 750 ppm DC group compared with the DEN alone group. However, significant increases in the number of γ-glutamyltranspeptidase (GGT)-positive cells and formation of microsomal ROS were not observed in the DEN + DC groups compared with the DEN alone group. Real-time polymerase chain reaction (RT-PCR) showed that the expression of *Cyp1a1*, *Cyp1a2*, and *OGG1*genes was significantly up-regulated in mice given diets containing 375 ppm DC or more, 187.5 ppm DC or more, and 750 ppm DC, respectively. These results suggest that the threshold dose of DC that induces ROS-mediated liver tumor promotion in mice is more than 750 ppm, although expression of the *Cyp1a2* gene, which is related to ROS generation, was up-regulated in the liver of mice, even at a DC dose of 187.5 ppm.

Key words: Dicyclanil, ROS generation, Threshold dose

INTRODUCTION

It has been shown that oxidative stress is an important factor that affects the pathogenesis of degenerative and inflammatory diseases, aging, and cancer (Wiseman and Halliwell, 1996; Trush and Kensler, 1991). Indeed, reactive oxygen species (ROS) are believed to play a pivotal role in the etiology of liver cancer, and ROS overproduction and subsequent oxidative DNA damage enhance the development of hepatocellular carcinomas caused by carcinogenic agents, such as the hepatitis C virus (Moriya *et al.*, 2001), metal (Asare *et al.*, 2006), insecticides (Lin *et al.*, 2007; Schilezinger *et al.*, 1999), and synthetic derivatives of naturally occurring flavonoids (Dewa *et al.*, 2008). In addition, it has been reported that upregulation of c-Myc resulting from the excessive ROS production may play an important role on cellular proliferation in the liver tumor promotion (Kawai *et al.*, 2009).

Dicyclanil (DC), 4,6-diamino-2-cyclopropylamino-pyrimidine-5-carbonitrile, is a pyrimidine-derived insect growth regulator that inhibits the molting and development of insects and is used in veterinary medicine to prevent myiasis (fly strike) in sheep. It has been reported that the incidence of hepatocellular carcinomas was increased in mice fed a diet containing 1,500 ppm of DC for 18 months, but negative results were obtained in *in vitro* and *in vivo* genotoxicity studies of DC (WHO, 2000). Based on these results, this chemical substance is classified as a nongenotoxic rodent carcinogen (WHO, 2000). In our previous study, to clarify the possible mechanism of DC-induced liver tumor promoting effects, we performed short- and long-term two-stage hepatocarcinogenesis

Correspondence: Kunitoshi Mitsumori (E-mail: mitsumor@cc.tuat.ac.jp)

studies in which mice were given 1,500 ppm DC after initiating hepatocarcinogenesis with N-dimethylnitrosamine (DMN) (Moto *et al.*, 2005, 2006b). The results of these two-stage hepatocarcinogenesis studies showed up-regulation of cytochrome P450 1A1 and cytochrome P450 1A2 (*Cyp1a1* and *Cyp1a2*) and thioredoxin reductase 1 (*Txnrd1*), which are metabolism- and/or oxidative stress-related genes, 8-oxoguanine DNA glycosylase (*OGG1*) and excision repair cross-complementing rodent repair deficiency group 5 (*Ercc5*), which are DNA damage-/repair-related genes (Moto *et al.*, 2005). Moreover, there were significant increases in the number of altered foci that were positive for γ-glutamyltranspeptidase (GGT-positive foci) and in the liver DNA levels of 8-hydroxydeoxyguanosine (8-OHdG), a representative marker of oxidative DNA damage, in mice of the DMN + DC group. In addition, a dose-dependent increase in microsomal ROS production was observed in these mice (Moto *et al.*, 2005, 2006b). These results suggest that DC has the potential to generate ROS via metabolic pathways and induce oxidative stress, including oxidative DNA damage, resulting in the induction of hepatocellular tumors in mice. However, the threshold dose of the liver tumor-promoting effect of DC in mice has not yet been elucidated. Additionally, in our previous study, real-time RT-PCR analysis revealed increased expression of the *Cyp1a1* gene and of phase II enzyme genes such as *Txnrd1* and *OGG1* in mice administered DC after an intraperitoneal injection of DMN compared with the gene expression in the corresponding control group (Moto *et al.*, 2006b). *Cyp1a1* generates ROS as byproducts of microsomal oxidation, and phase II enzymes are involved in the protection against oxidative stress (Guengerich and Shimada, 1991; Puntarulo and Cederbaum, 1998). Thus ROS generation and protection against ROS by phase II enzymes probably occur concomitantly in mice administered DC after an intraperitoneal injection of DMN. Accordingly, it is considered that the dose increasing ROS generation, cell proliferative activity, GGT positive foci or cell and drug-metabolizing enzymes that protect against ROS is the threshold dose of liver tumor promotion in mice. However, the threshold dose of DC in mice is not known.

In our previous studies, the incidence of hepatocellular tumors (adenomas and carcinomas) significantly increased in partially hepatectomized male ICR mice that were intraperitoneally injected with N-diethylnitrosamine (DEN) and given a diet containing 1,500 ppm of DC for 20 weeks, and gene expression analysis on the micro-dissected liver tissues of the mice in this group showed the highest expression levels of oxidative stress-related genes, such as *Cyp1a1* and *Txnrd1*, in the hepatocellular tumor areas (Moto *et al.*, 2006a). Therefore, in the present study, we selected DEN rather than DMN as an initiator, since it was confirmed that mice were more susceptible to DEN than DMN in our preliminary studies (personal observations). In addition, we used a two-stage hepatocarcinogenesis model in mice to identify the threshold dose of DC that induces liver tumor-promoting effects, microsomal ROS generation and drug-metabolizing enzymes that protect against ROS.

MATERIALS AND METHODS

Animals and chemicals

Four-week-old male ICR mice were purchased from Japan SLC, Inc. (Shizuoka, Japan) and were maintained on a powdered basal diet (MF; Oriental Yeast, Co., Ltd., Tokyo, Japan) and tap water until they were 5 weeks old. The mice were housed in polycarbonate cages with paper bedding and were maintained under standard conditions (room temperature, 22 ± 2°C; relative humidity, 55 ± 5%; light/dark cycle, 12 hr). Animal care and experiments were carried out in accordance with the Guide for Animal Experimentation of the Tokyo University of Agriculture and Technology.

DC (CAS No. 112636-83-6) was kindly provided by Novartis Animal Health Inc. (Basel, Switzerland) for the experiment. DEN was purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

Experimental design

The two-stage liver carcinogenesis model of mice was used based on a modified protocol of Della Porta *et al.* (1987) and Lee *et al.* (1989) (Fig. 1). To initiate hepatocarcinogenesis, an intraperitoneal injection of DEN at a dose of 30 mg/kg body weight was administered to the animals (day 0). Twenty-four hours before the DEN injection, a two-thirds partial hepatectomy was performed on the mice to enhance the regeneration of the liver with DNA damage. One week after the DEN injection, mice were fed a powdered diet containing DC at a concentration of 0 (DEN alone), 187.5, 375 or 750 ppm for 10 weeks. For liver sampling, the survivors at 11 weeks were sacrificed by exsanguination from the abdominal aorta under ether anesthesia.

At necropsy, tissue samples were collected from the remaining lobes of the liver. One-third of these samples were fixed with natural-buffered formalin for histopathological examinations, and one-third was embedded in OCT compound (Tissue-Tek; Miles Inc., Elkhart, IN, USA) and frozen for GGT staining. The remaining liver samples were weighed, frozen in dry ice and stored at –80°C until

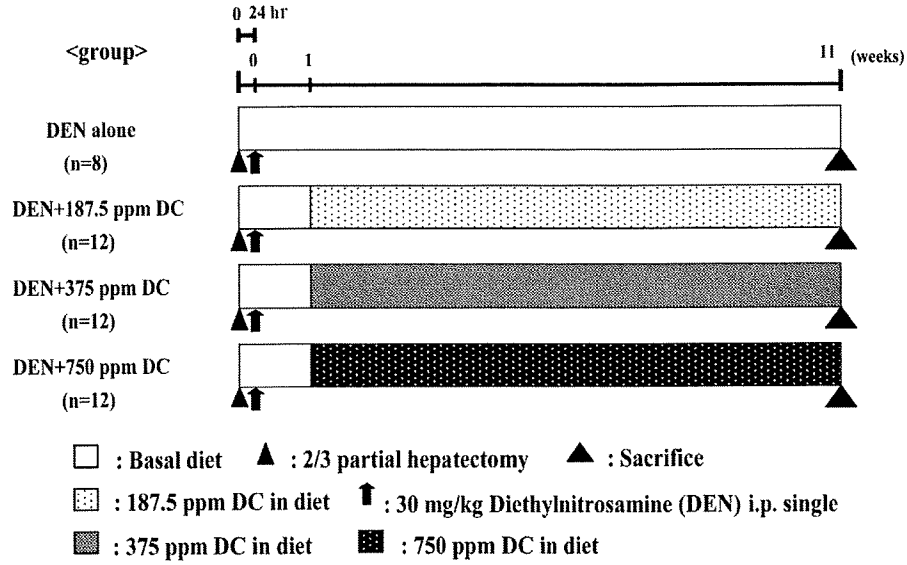

Fig. 1. Experimental design

further analysis.

Histopathological, histochemical and immunohistochemistry evaluations

Formalin-fixed liver tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (HE) for the histological examinations. Immunohistochemical staining of proliferating cell nuclear antigen (PCNA) antibody (PC10; DakoCytomation, Glostrup, Denmark) was performed by the avidin-biotin-peroxidase complex method. Histochemical staining of GGT was performed by modifying the methods proposed by Rutenberg *et al.* (1969). The frozen tissues were cryosectioned and fixed using acetone. After air-drying, the freshly prepared solution containing the substrate L-glutamic acid-γ-(4-metoxy-β-naphthylamide) (Sigma-Aldrich, St. Louis, MO, USA) and fast blue BBN (Wako Pure Chemical Industries, Osaka, Japan) in 0.1 M Tris-buffered saline (pH 7.4) was coated onto the section. After incubation, the slides were transferred to 0.1 M cupric sulfate solution. The sections were then stained with hematoxylin, and mounted in 10% glycerol. The number of PCNA-positive cells per 400-500 cells in each slide was counted from ten different areas to determine the PCNA positive index (PCNA PI). The number of GGT-positive cells per area was calculated from the number of positive cells in all lobes on the slide and from the total area in all lobes measured using computer-assisted image analyzer (NIH image).

RNA isolation and real-time RT-PCR

Total RNA was isolated from the liver samples using TRIzol reagent (Invitrogen Corp., Carlsbad, CA, USA). Reverse transcription (RT) was carried out using ThermoScript RT-PCR System (Invitrogen Corp.), and the cDNA aliquots were used in quantitative real-time RT-PCR with SYBR Green using an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The PCR reaction was repeated twice in triplicate for each gene. The PCR primers for the genes of cytochrome P450 1A1 (*Cyp1a1*, accession number NM_009992), cytochrome P450 1A2 (*Cyp1a2*, accession number NM_009993), 8-oxoguanine DNA-glycosylase 1 (*OGG1*, accession number NM_010957), thioredoxin reductase 1 (*Txnrd1*, accession number NM_015762), aldehyde dehydrogenase family 1 subfamily A1 (*Aldh1a1*, accession number NM_013467), NADPH P450 reductase (*Por* or NADPH, accession number NM_008898), Excision repair cross-complementing rodent repair deficiency complementation group 5 (*Ercc5*, accession number NM_011729), superoxide dismutase 1 (*Sod1*, accession number NM_128337) and Glutathione peroxidase 2 (*Gpx2,* accession number MN_030677) in the present study were prepared as previously reported (Moto *et al.*, 2005, 2006a and 2006b). The primers used in this PCR are summarized in Table 1. The expression levels of each gene were corrected based on the expression of β-actin in the same cDNA sample.

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

Accession number	Abbreviation of gene name	Primer (upper: forward primer, lower: reverse primer)
NM_009992	*Cyp1a1*	AGGATGTGTCTGGTTACTTG
		AGAAACATGGACATGCAAG
NM_009993	*Cyp1a2*	GCTACTTGTGACATGGCCTA
		AAGCCATTCAGTGAGGTGTC
NM_010957	*Ogg1*	CAGCATAAGGTCCCCACAGATT
		GCCAACAAAGAACTGGGAAACT
NM_015762	*Txnrd1*	GGTTGCATACCTAAGAAGCTGATG
		CCATAGTTGCGCGAGTCTTTC
NM_013467	*Aldh1a1*	GACTTGAAGATTCAACATACC
		TCACAGCTTTGTCAACATCA
NM_008898	*Por or NADPH*	GCCTGCCTGAGATCGACAAG
		GGGTCGCCTTCTCCGTATGT
NM_011729	*Ercc5*	TCAACTAGGACTGGACCGTAACAA
		AGTTGGTATCCCTTCCGTATAGTCA
NM_128337	*Sod1*	TGATTGGGATTGCGCAGTAA
		TGGTTTGAGGGTAGCAGATGAGT
NM_030677	*Gpx2*	GCTGCCCTACCCTTATGATGAC
		CGCACGGGACTCCATATGAT

Measurement of microsomal ROS production in the liver

Liver microsomes were obtained from four mice from each group treated with 0, 187.5, 375 or 750 ppm DC for 10 weeks and which were subjected to the two-stage hepatocarcinogenesis model. Their liver microsomes were extracted using the following method. The liver samples were rinsed with ice-cold homogenate buffer (1.15% KCl, 0.2 mM ethylene diamine tetra-acetic acid disodium (EDTA-2Na), 0.1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 20% glycerin at pH 7.4) and homogenized in three volumes of the homogenate buffer using a glass pestle. The homogenate was centrifuged at 10,000 × g for 10 min, and the supernatant was ultracentrifuged at 105,000 × g for 90 min. The microsomal pellet was resuspended in the homogenate buffer, and the protein content of the homogenate was determined using the BCA Protein Assay Kit (Pierce Biotechnology, Pierce, IL, USA) with BSA as a standard. For the measurement of ROS, the nonfluorescent probe DCFH-DA was used as to detect ROS formation during cellular metabolism; in the presence of ROS, this probe oxidizes to a highly fluorescent dichlorofluorescein (DCF) (LeBel *et al.*, 1990; Serron *et al.*, 2000; Szejda *et al.*, 1984). The microsomes (0.05 mg protein) were incubated in the dark at 37°C in 40 mM Tris buffer (pH 7.4) and DCFH-DA (5 μM) for 30 min. At the end of the incubation period, 2.5 mM β-NADPH was added and the sample was incubated at 37°C for 30 min in the dark. ROS formation was detected by measuring the fluorescent product by a microplate reader (excitation, 485 nm and emission, 528 nm).

Statistical evaluation

Statistical analyses were performed using the statistical software StatLight (Yukms Co., Ltd., Tokyo, Japan), and all results are presented as means ± S.D. The significance of the differences between the DEN alone group and each DC group was determined by Dunnett's test after one-way ANOVA. A p-value less than 0.05 was considered statistically significant in both analyses.

RESULTS

General observations and histological evaluation

During the experimental period, death and remarkable treatment-related clinical signs were not observed in the DEN alone or the DEN + DC groups. No significant differences in the final body weight, and absolute and relative liver weights of mice were observed in any of the treated groups (Table 2, Fig. 2).

No remarkable histopathological changes were observed in the liver of mice treated with 187.5, 375 or 750 ppm DC. In immunohistochemical examinations, GGT-positive cells were observed in the liver of mice treated with DEN alone or DEN + DC, but GGT-positive foci consisting of the cluster of GGT-positive cells were not observed in these groups. The number of GGT-positive cells was not significantly increased in the DEN + DC groups compared with that in the DEN alone group (Fig. 3a). However, the PCNA-positive cell ratio was significantly increased in the 750 ppm DC group compared with that in the DEN alone group (Fig. 3b).

mRNA expression in liver tissues

The results of the analyses are summarized in Fig. 4. A significant up-regulation of stress response-related genes such as *Cyp1a1* and *Cyp1a2* was observed in mice administered 375 ppm DC or more and mice in the DC-treated groups as compared with the control group, respectively. The expression of *OGG1*, a DNA related gene, was significantly up-regulated only in the 750 ppm DC group compared with the control group. No significant differences were found in the expression of Heme oxygenase 1 (*Hmox1*, MN_010442), *Ercc5*, *Por* (NADPH), *Txnrd1*, *Sod1*, *Gpx2*, and oxidative stress-related genes between the DEN alone and DEN + DC groups.

Table 2. Body and liver weights of mice given DC after DEN initiation

Group	DEN alone	DEN+DC 187.5 ppm	DEN+DC 375 ppm	DEN+DC 750 ppm
Animal No.	7	12	12	10
Body weight (g)	43.8 ± 6.5[a)]	43.5 ± 4.0	42.2 ± 4.0	41.4 ± 3.2
Absolute liver weight (g)	2.0 ± 0.2	2.2 ± 0.9	1.9 ± 0.2	2.0 ± 0.4
Relative liver weight (%)	4.7 ± 0.2	5.0 ± 1.7	4.7 ± 0.5	4.8 ± 0.8

[a)]: Mean ± S.D.

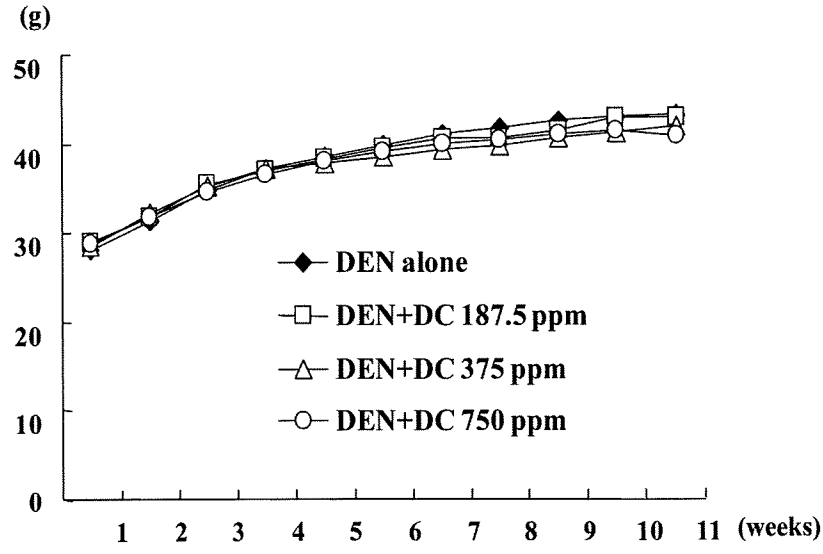

Fig. 2. Body weight changes of mice given DC for 10 weeks after DEN initiation

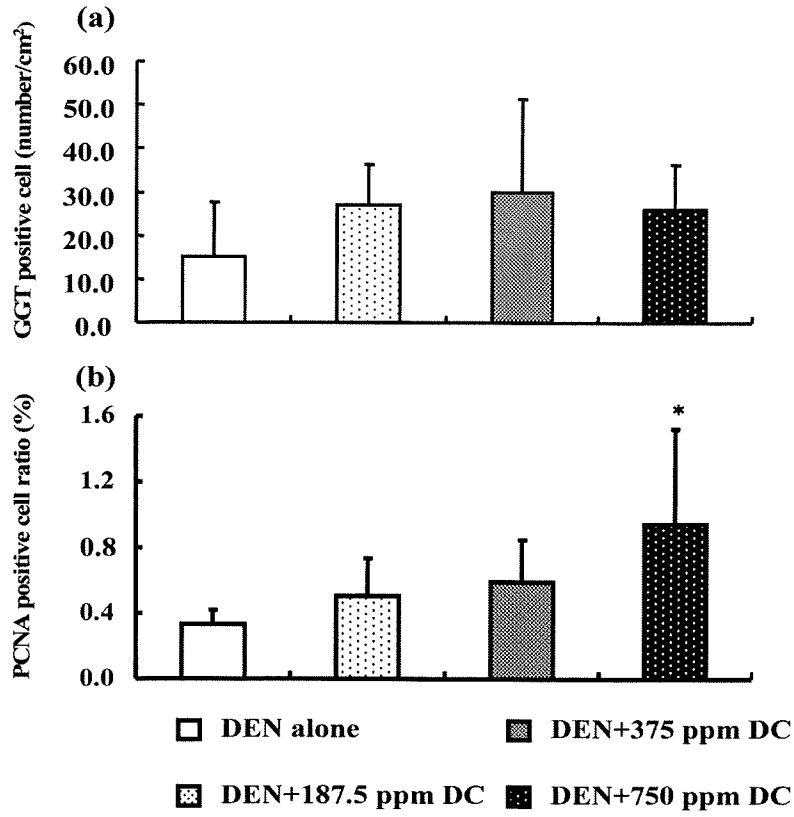

Fig. 3. The number of GGT positive cells and the PCNA positive ratio in mice given DC for 10 weeks after DEN initiation. Each graph shows the number of GGT-positive cells (a) and the PCNA-positive cell ratio (b) in the livers of mice given DC for 10 weeks after DEN initiation. Column represents means ± S.D. *Significantly different from the 0 ppm DC group at $p < 0.05$ (Dunnett's test).

Formation of microsomal ROS in liver

As shown in Fig. 5, there was a tendency for increased formation of microsomal ROS in the liver of the DC-treated groups, but there were no significant differences between the DEN alone and DEN + DC groups.

DISCUSSION

DC has been reported to potentially exert hepatocarcinogenic and hepatocellular tumor-promoting effects in mice (WHO, 2000; Moto *et al.*, 2005, 2006a and 2006b). In our previous study, we performed a two-stage liver carcinogenesis experiment in which partially hepatectomized mice were given 1,500 ppm DC for 8, 13 or 20 weeks after initiation of hepatocarcinogenesis by DMN or DEN, and the number and area of GGT-positive foci and PCNA-positive ratio were significantly increased in mice of the DMN + DC or DEN + DC group compared with those in the DMN or DEN alone group (Moto *et al.*, 2005, 2006a and 2006b). GGT is used as a marker for preneoplastic lesions in chemically induced hepatocarcinogenesis of mice (Peraino *et al.*, 1983). In the present study, there was no increase in the number of GGT-positive cells in the livers of mice administered DC compared with that in the DEN alone group. However, immunohistochemical findings revealed a significant increase in the PCNA-positive ratio in the 750 ppm DC group compared with that in the DEN alone group. It is has been reported that the amount of PCNA positive cells were corresponding with proliferative condition of the hepatic proliferative lesions (Chen *et al.*, 1998). Furthermore, there are many reports that PCNA positive cell significantly increased in the liver of mice with the increased number of GGT positive foci or cells (Moto *et al.*, 2005, 2006a and 2006b; Kenmochi *et al.*, 2007). These results suggested the possibility that the present concentration of DC is not enough to the forma-

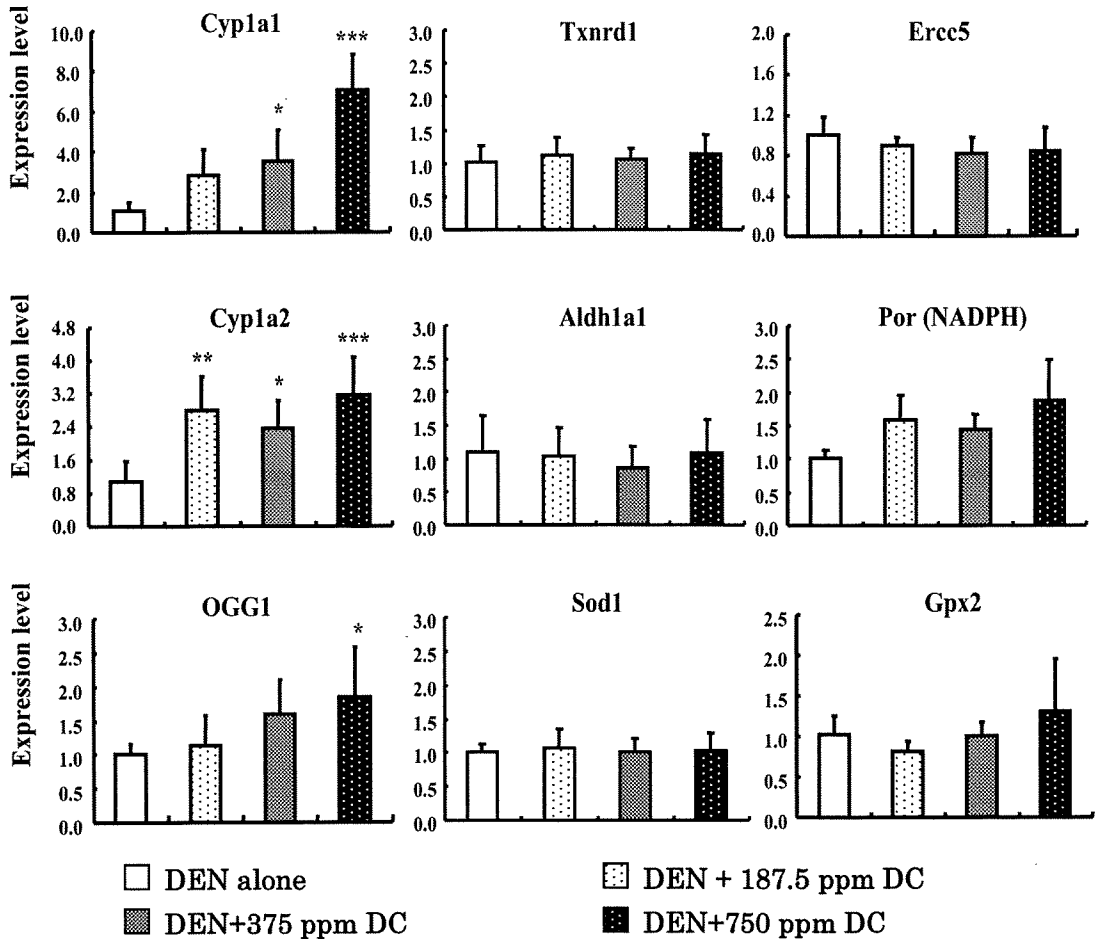

Fig. 4. Real time RT-PCR analysis of mRNA expression in the livers of mice given DC for 10 weeks after DEN initiation. The expression of target genes was normalized by β-actin. Values are expressed as the group mean fold change over control. Columns represents means ± S.D. *, ** and *** significantly different from the 0 ppm DC group at $p < 0.05$, $p < 0.01$ or $p < 0.001$, respectively (Dunnett's test).

tion of preneoplastic lesions, although an increase in cell proliferation activity was observed in the liver of mice given 750 ppm DC. Accordingly, the practical threshold level of DC that induces hepatocellular tumor-promoting effects was considered to be more than 750 ppm in the present experimental condition.

The cytochrome P-450 family is known to generate ROS as byproducts of microsomal oxidation. Among the CYPs, CYP1A1 has been reported to be the most active CYP enzyme that catalyzes procarcinogens (Guengerrich and Shimada, 1991; Puntarulo and Cederbaum, 1998), and the up-regulation of the CYP1A1 and/or CYP1A2 isoform(s) indirectly results in the production of very large amounts of ROS compared with that of other CYPs (Paolini *et al.*, 1996; Putarulo and Cederbaum, 1998; Valgimigli *et al.*, 2001). In our previous studies in which mice were given diet containing 1,500 ppm DC after DMN/DEN initiation, a dose-dependent increase in microsomal ROS production was observed in these treated mice (Moto *et al.*, 2005, 2006b). In the present study, the expressions of *Cyp1a1* in mice given 375 ppm DC or more and *Cyp1a2* in mice from all of the DC-treated groups were significantly and higher than that in the DEN alone group, although there were no significant differences in the formation of microsomal ROS between the DEN alone and DEN + DC groups. These findings may suggest a possible generation of ROS through P450-mediated metabolism of DC and that the ROS produced in the liver of mice given DC is derived from the microsomes. On the other hand, it is also well known that ROS gen-

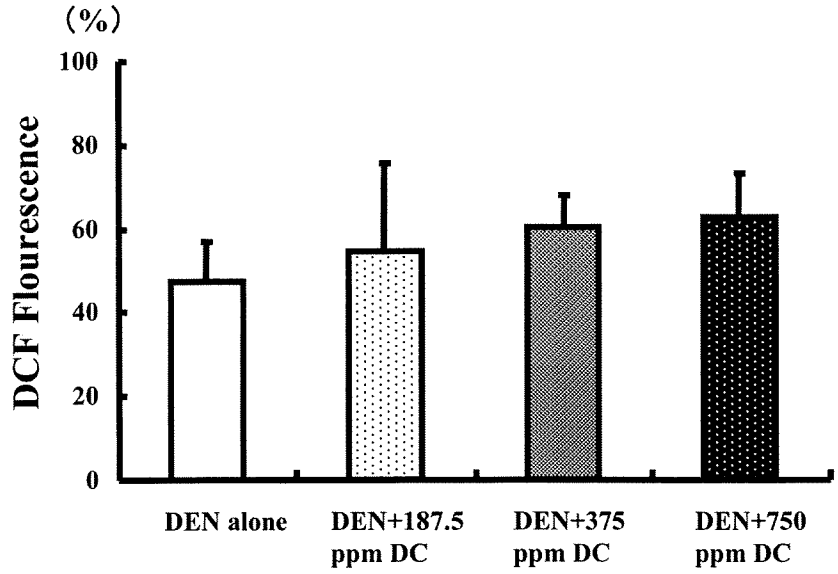

Fig. 5. Effect of DC on hepatic microsomal ROS production. Column represents mean ± S.D.

erates from other cellular organelle in hepatocytes, such as mitochondria and peroxisomes (Parola and Robino, 2001), and macrophages (Kupffer cells) (Loguercio and Federico, 2003). However, no morphological changes were observed in Kupffer cells as well as mitochondria and peroxisomes of hepatocytes in DC-treated mice in our previous and present studies (Moto, *et al.*, 2005, 2006a and 2006b). Therefore, the possibility that ROS generated from these organelles and Kupffer cells in DC-treated mice can be eliminated.

In the preset study, the formation of microsomal ROS in the liver tended to increase in the DC-treated groups, but there were no significant differences between the DEN alone and DC-treated groups. This finding may suggest that no oxidative DNA damage occurs in mice given 750 ppm DC or lower. On the other hand, the expression of *OGG1* was significantly increased only in the 750 ppm DC group compared with that in the control group. *OGG1* is a repair gene of 8-OHdG (Kinoshita *et al.*, 2002, 2003). 8-OHdG, a marker of oxidative DNA damage, is known as one of the causes for DNA point mutations such as G-T transversion (Kasai, 1997; Shibutani *et al.*, 1991), and is potentially involved in the carcinogenesis in various experimental models (Nakae *et al.*, 1997; Yoshida *et al.*, 1999; Kinoshita *et al.*, 2002, 2003). The up-regulation of the *OGG1* gene in the present study indicates that oxidative DNA damage in mice given 750 ppm DC may be prevented by this DNA repair gene. On the other hand, the mRNA expression of metabolism-, oxidative stress- or DNA damage-/repair-related genes, such as *Txnrd1*, *Por*, *Aldh1a1*, *Sod1*, *Gpx2* and *Ercc5*, in the DC-treated groups was not significantly higher than that in the DEN alone group. These findings may indicate that neither oxidative stress nor DNA damage is induced in mice given DC at concentrations of 750 ppm or lower. Taking into account our results, we speculate that *Cyp1a1* and *Cyp1a2* are the most sensitive molecular markers of the treatment-related metabolic changes of DC, but the up-regulation of these genes at a concentration of DC of 750 ppm or lower is not enough to produce ROS.

In conclusion, in the present study, an increased number of PCNA-positive cells, and enhanced expression of oxidative stress- or DNA damage-/repair-related genes such as *Cyp1a1*, *Cyp1a2* and *OGG1* were observed in the livers of mice given 750 ppm DC compared with the DEN alone group, but the number of GGT-positive cells and formation of microsomal ROS were not increased in this group. Therefore, the results of our study suggest that the threshold dose for ROS-mediated liver tumor promotion of DC is more than 750 ppm. Accordingly, the possibility that hepatocellular proliferative lesions may be induced in mice given 750 ppm DC or lower is extremely low.

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GENOTOXICITY AND CARCINOGENICITY

Elevation of cell proliferation via generation of reactive oxygen species by piperonyl butoxide contributes to its liver tumor-promoting effects in mice

Masaomi Kawai · Yukie Saegusa · Yasuaki Dewa · Jihei Nishimura · Sayaka Kemmochi · Tomoaki Harada · Yuji Ishii · Takashi Umemura · Makoto Shibutani · Kunitoshi Mitsumori

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Abstract Piperonyl butoxide (PBO) is a pesticide synergist used with pyrethroids as a domestic insecticide, and it acts as a non-genotoxic hepatocarcinogen in rats and mice. To clarify whether oxidative stress is involved in the liver tumor-promoting effect of PBO in mice, male mice were subjected to two-thirds partial hepatectomy, followed by *N*-diethylnitrosamine (DEN) treatment, and given a diet containing 0.6% PBO for 25 weeks. The incidences of cytokeratin (CK) 8/18-positive foci, adenomas, and carcinomas significantly increased in the DEN + PBO group compared with the DEN-alone group. The PCNA-positive ratio significantly increased in non-tumor hepatocytes, CK8/18-positive foci and adenomas in the DEN + PBO group compared with the DEN-alone group. PBO increased reactive oxygen species (ROS) production in microsomes but did not change oxidative DNA damage as assessed by 8-hydroxydeoxyguanosine (8-OHdG). In real-time RT–PCR, PBO upregulated the expression of genes related to metabolism, such as *Cytochrome P450 1a1*, *2a5*, and *2b10*, and metabolic stress, such as *Por* and *Nqo1*, but downregulated *Egfr* and *Ogg1*. PBO also increased early response genes downstream of mitogen-activated protein kinase (MAPK), such as *c-Myc* that is induced by excessive ROS production, and G1/S transition-related genes, such as *E2f1* and *Ccnd1*. Thus, PBO can generate ROS via the metabolic pathway without any induction of oxidative DNA damage, activate cell growth, increase c-Myc- and E2F1-related pathways, and act as a liver tumor promoter of DEN-induced hepatocarcinogenesis in mice.

Keywords Piperonyl butoxide · Oxidative stress · Mitogen-activated protein kinase · 8-hydroxydeoxyguanosine · Hepatocarcinogenesis

Abbreviations

PBO	Piperonyl butoxide
DEN	*N*-diethylnitrosamine
RT–PCR	Reverse transcription-polymerase chain reaction
CK	Cytokeratin
ROS	Reactive oxygen species
8-OHdG	8-hydroxydeoxyguanosine
Cyp1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1
Cyp2a5	Cytochrome P450, family 2, subfamily a, polypeptide 5
Cyp2b9	Cytochrome P450, family 2, subfamily b, polypeptide 9
Cyp2b10	Cytochrome P450, family 2, subfamily b, polypeptide 10
Ogg1	8-xoguanine DNA glycosylase
Por	P450 (cytochrome) oxidoreductase
Nqo1	NAD(P)H dehydrogenase, quinone 1
MAPK	Mitogen-activated protein kinase
c-Myc	Myelocytomatosis oncogene
E2f1	E2F transcription factor 1

M. Kawai · Y. Saegusa · Y. Dewa · J. Nishimura · S. Kemmochi · T. Harada · M. Shibutani · K. Mitsumori (✉)
Laboratory of Veterinary Pathology,
Tokyo University of Agriculture and Technology,
3-5-8 Saiwai-cho, Fuchu-shi, Tokyo 183-8509, Japan
e-mail: mitsumor@cc.tuat.ac.jp

M. Kawai · Y. Saegusa · Y. Dewa · J. Nishimura · S. Kemmochi
Pathogenetic Veterinary Science,
United Graduate School of Veterinary Sciences,
Gifu University, Gifu 501-1193, Japan

Y. Ishii · T. Umemura
Division of Pathology, National Institute of Health Sciences,
18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

ccnd1	Cyclin D1
EGFR	Epidermal growth factor receptor
GGT	Gamma-glutamyl transpeptidase
GST-P	Glutathione s-transferase placental form
DAB	3,3′-diaminobenzidine
DEN	Diethylnitrosamine
DCFH-DA	2′7′-Dichlorodihydro-fluorescein diacetate
β-NADPH	β-Nicotinamide adenine dinucleotide phosphate
PCNA	Proliferating cell nuclear antigen
PBS	Phosphate buffered saline
HE	Hematoxylin and eosin
NADPH	Nicotinamide adenine dinucleotide phosphate
CT	Cycle time

Introduction

Many chemicals, such as drugs, agricultural chemicals, and food additives, generate reactive oxygen species (ROS) via metabolic pathways and induce oxidative stress. ROS are required for physiologic function and regulate cellular signaling in normal cells. However, excessive ROS can induce DNA damage and cause genomic instability, which may contribute to cancer progression. Thus, ROS play multiple roles in tumor initiation, progression, and maintenance (Benhar et al. 2002). We have reported that oxidative stress promotes tumors in rats after treatment with oxfendazole, fenbendazole, fenofibrate, and piperonyl butoxide (PBO), from the early stage of a two-stage hepatocarcinogenesis model (Muguruma et al. 2007; Dewa et al. 2009; Nishimura et al. 2007; Taniai et al. 2009). Our data suggest that these chemicals induce sustained oxidative stress in the livers, which accounts for the enhancement of microsomal ROS production and increased oxidative DNA damage.

PBO is a pesticide synergist that is widely used with pyrethroids for grain protection and as a domestic insecticide. PBO is a non-genotoxic hepatocarcinogen in F344 rats fed a diet containing 1.2% PBO for 2 years (Takahashi et al. 1994) and CD-1 mice fed a diet containing 300 mg/kg/day PBO for 79 weeks (Butler et al. 1998). We previously showed that the number of glutathione *S*-transferase placental form (GST-P)-positive foci and the expression of *Cyp1a1* gene significantly increased in rats given a diet containing 1 or 2% PBO (Muguruma et al. 2007). The cytochrome P-450 family generates ROS as byproducts of microsomal oxidation, and the upregulation of CYP1A1 and 1A2 isoforms indirectly results in the production of large amounts of ROS (Puntarulo and Cederbaum 1998; Nishikawa et al. 2002). Moreover, PBO and phenobarbital induce CYP2B1 and inhibit intercellular communication through gap junctions by downregulating connexin 32 in the liver of rats (Okamiya et al. 1998). Moreover, the formation of 8-hydroxydeoxyguanosine (8-OHdG), a sensitive marker of oxidative DNA damage (Kasai 1997; Valavanidis et al. 2009), increased in the liver of rats given a diet containing 2% PBO in a two-stage hepatocarcinogenesis model (Muguruma et al. 2007). Therefore, PBO can generate ROS via a metabolic pathway in the liver of rats and induce oxidative stress, including oxidative DNA damage (Muguruma et al. 2009). On the contrary, in male ICR mice fed a diet containing 0.6% PBO for 8 weeks without any initiation treatment, microarray analysis and real-time RT–PCR revealed that oxidative and metabolic stress-related genes, such as *Cyp1a1*, *Cyp2a5*, *Cyp2b9*, *Cyp2b10*, and NADPH-cytochrome P450 oxidoreductase (*Por*), were upregulated (Muguruma et al. 2006). Therefore, PBO could also generate ROS via the metabolic pathway and induce oxidative stress that is possibly related to the hepatocarcinogenesis in mice. However, there is no data demonstrating that oxidative damage resulting from ROS generation induced by the long-term PBO treatment is involved in the liver tumor promotion of hepatocarcinogenesis in mice.

Here, we investigated the potential mechanism underlying the liver tumor-promoting effect of PBO in mice, with particular attention on gene expression and biochemical events affecting the cell cycle regulators, using a DEN-initiated hepatocarcinogenesis model in partially hepatectomized mice given PBO for 25 weeks.

Materials and methods

Chemicals

PBO, α-[2-(2-butoxyethoxy)ethoxy]-4,5-methylenedioxy-2-propyltoluene (CAS register number 51-03-6, EU Number PBO, purity 90%), was purchased from Kanto Chemical Co., Inc. (Kumamoto, Japan). 3,3′-diaminobenzidine (DAB) was purchased from Dojindo Molecular Technologies, Inc. (Tokyo, Japan). Diethyl nitrosamine (DEN) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Anti-cytokeratin 8/18 polyclonal antibody was purchased from PROGEN Biotechnik GmbH (Heidelberg, Germany). Monoclonal mouse anti-proliferating cell nuclear antigen (PCNA) antibodies were purchased from DAKO Cytomation A/S (Glostrup, Denmark). Trizol reagent, SuperScript™ III reverse transcriptase, and random primers were purchased from Invitrogen Corporation (Carlsbad, CA, USA). The Power SYBR Green PCR Master Mix was purchased from Applied Biosystems (Foster City, CA, USA). The BCA Protein Assay Kit was purchased from Pierce Biotechnology (Rockford, IL, USA). 2′7′-Dichlorodihydro-fluorescein diacetate (DCFH-DA) was purchased from Molecular Probes Inc.

(Eugene, OR, USA). β-Nicotinamide adenine dinucleotide phosphate (β-NADPH), dithiothreitol (DTT), and Protease Inhibitor Cocktail were purchased from Sigma–Aldrich, Inc. (St Louis, MO, USA). All other reagents were commercially available and of analytical grade.

Animals and experimental design

Six-week-old male ICR mice were obtained from Japan SLC Inc. (Shizuoka, Japan). They were housed in plastic cages (five animals/cage) with absorbent paper chip bedding in an animal room maintained under standard conditions (room temperature, 22 ± 2°C; relative humidity, 55 ± 5%; and light/dark cycle, 12 h) and given free access to a powdered diet (Oriental MF; Oriental Yeast, Tokyo, Japan) and tap water. The animals were acclimatized for 1 week prior to beginning the experiment. The experiment was performed in accordance with the guidelines for animal experimentation of the Tokyo University of Agriculture and Technology. We used a short-term, two-stage liver carcinogenesis model (Moto et al. 2006) in ICR mice. To enhance hepatocellular proliferation, mice were subjected to a two-thirds partial hepatectomy. Twenty-four hours after the hepatectomy, mice were given a single i.p. injection of DEN (20 mg/kg body weight) dissolved in saline to initiate hepatocarcinogenesis. One week after injection, the animals were subdivided into two groups and given a powdered diet containing 0 or 0.6% PBO for 25 weeks. On completion of treatment, the mice were killed by exsanguination from the posterior vena cava under ether anesthesia, and livers were immersed in 4% paraformaldehyde solution for microscopy. Some of the livers cut into small pieces without the macroscopic nodules, quickly frozen in liquid nitrogen, and stored at −80°C until analysis.

Histopathology, immunohistochemistry, and quantitative analysis

After sacrifice, livers were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned at 3-μm thickness, and stained with hematoxylin and eosin (HE) for histopathological examinations. The incidence of liver proliferative lesions, such as altered foci, adenomas, and carcinomas, in two HE-stained sections/mouse was counted under a light microscope.

For immunohistochemistry, paraffin-embedded liver sections were deparaffinized in xylene and rehydrated in ethanol. For antigen retrieval, the liver sections for PCNA were incubated with citrate buffer [0.1 mol/L citrate (pH 6.0)] and heated in a microwave oven at low power for 30 min before incubation with 0.3% hydrogen peroxide in PBS. These liver sections were subjected to immunohistochemistry using a mouse-on-mouse immunodetection kit (Vector Laboratories Burlingame, CA, USA) for PCNA. The specimens were incubated overnight with the PCNA antibody at a dilution of 1:300 in 0.5% casein–PBS at 4°C.

The liver sections for CK8/18 incubated with citrate buffer [0.1 mol/L citrate (pH 6.0)] and heated in a microwave oven at 98°C for 30 min before incubation with 0.3% hydrogen peroxide in PBS. Non-specific binding sites were blocked with blocking normal goat serum. The specimens were incubated overnight with the CK8/18 antibody at a dilution of 1:100 in 0.5% casein–PBS at 4°C. The sections were incubated with a guinea pig peroxidase-conjugated secondary antibody (Fitzgerald Industries International Inc., MA, USA) diluted in PBS supplemented with 0.5% casein. Subsequently, 3, 3′-diaminobenzidine (DAB, Dojindo laboratories, Kumamoto, Japan) was applied as a chromogen. The sections were finally counterstained with hematoxylin.

The number of CK8/18-positive foci, adenoma, and carcinoma, and the total areas of liver sections were estimated using WinROOF image analysis software (version 5.7; Mitani Corp., Fukui, Japan), and the number of foci per unit area (cm^2) of liver sections was calculated. The PCNA-positive indices were estimated for CK8/18-positive areas using WinROOF image analysis software package.

Measurement of microsomal ROS production in the liver

The liver samples from each treatment group were homogenized with three volumes of ice-cold homogenate buffer (1.15% KCl, 0.2 mM EDTA-2Na, 0.1 mM DTT, 0.1% protease inhibitor cocktail, 20% glycerin; pH 7.4) using a glass-Teflon homogenizer. The homogenate was centrifuged at 700×*g* for 10 min, and the supernatant was centrifuged at 10,000×*g* for 20 min. The resultant supernatant was further centrifuged at 105,000×*g* for 60 min. Finally, the pellet was resuspended in 0.25 mol/l sucrose and 0.05 mol/l Tris–HCl buffer (pH 7.4) as the microsomal fraction and stored at −80°C. Microsomal protein concentrations were determined using a BCA Protein Assay Kit (Pierce, IL, USA).

2′,7′-Dichlorodihydrofluoresceine diacetate (DCFH-DA; Molecular Probes Inc., Eugene, OR, USA) was used as a intracellular probe for detecting ROS formation during the metabolic process by converting DCFH-DA to the highly fluorescent 2′,7′- dichlorofluorescein through oxidation by ROS. Liver microsomes (0.05 mg protein) were incubated in the dark at 37°C in 40 mmol/l Tris buffer (pH 7.4) and DCFH-DA (5 mmol/l) for 30 min followed by further incubation for 30 min after addition of 0.6 mmol/l NADPH. The formation of ROS was detected by measurement of the fluorescent product using a fluorescence microplate reader (excitation, 485 nm; emission, 528 nm).