Preparation of microsomal fraction

The microsomal fractions were obtained according to the method of Yoshihara et al. (2001). Briefly, the liver samples from each treatment group were homogenised with three volumes of ice-cold 0.25 mol/l sucrose −0.05 mol/l Tris-HCl buffer (pH 7.4) using a glass-Teflon homogeniser. 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, and the resultant pellet was resuspended and centrifuged again at 105,000*g* for 60 min. Finally, the pellet was resuspended in the 0.25 mol/l sucrose−0.05 mol/l Tris-HCl buffer (pH 7.4) as the microsomal fraction and stored at −80°C. The microsomal protein concentrations were determined by a BCA Protein Assay Kit (Pierce, IL, USA).

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 method of Schlezinger et al. (1999). Briefly, 0.1 mg of microsomal protein was incubated with 5 μmol/l H_2DCFDA (Invitrogen) in Hank's balanced salt solution (Invitrogen) at 30°C for 15 min. After incubation, this microsomal solution was transferred into a 96-well plate. The reactions were initiated with 1.4 mmol/l NADPH (Wako Pure Chemical Industries), and the fluorescence was monitored over 5 min using the Synergy HT Multi-Detection Microplate Reader (BioTek, VT, USA) with excitation and emission wavelengths of 485 and 528 nm, respectively. In some cases, SKF-525A (Toronto Research Chemicals, ON, Canada), a well-known inhibitor of cytochrome P450, was added to the well at a final concentration of 0.1 mmol/l. DCF production (pmol min^{-1} mg $protein^{-1}$) was obtained from standard curves prepared using DCF.

Western blotting

A total of 20 μg of microsomal proteins was electrophoresed on a 10% sodium dodecyl sulphate (SDS) gel, transferred to a polyvinylidene difluoride (PVDF) membrane (Hybond-P; GE Healthcare), and blocked. The membrane was incubated with anti-CYP1A1 (1:400 dilution; Santa Cruz Biotechnology, CA, USA) or anti-CYP2E1 (1:1,000 dilution; Biomol International, PA, USA) overnight at 4°C, followed by incubation in goat anti-rabbit IgG (horseradish peroxidase (HRP)-conjugated, 1:10,000 dilution; GE Healthcare) for 2 h at room temperature. The immunoproducts were visualised using ECL Plus™ Western blotting detection reagents (GE Healthcare).

Determination of 8-OHdG and TBARS levels

Oxidative DNA damage and lipid peroxidation in the livers were estimated on the basis of the levels of 8-hydroxydeoxyguanosine (8-OHdG) and thiobarbituric acid-reactive substances (TBARS), respectively.

The 8-OHdG levels in liver DNA were determined using the method of Umemura et al. (2006). Briefly, nuclear DNA 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., MA, USA) according to the running condition previously reported (Umemura et al. 2006).

The levels of hepatic TBARS were determined using the method of Ohkawa et al. (1979). Briefly, 0.2 ml of liver homogenate in 1.15% KCl, 0.2 ml of 8.1% SDS and 3.0 ml of 0.4% thiobarbituric acid in 10% acetic acid (pH 3.5) were mixed, heated at 95°C for 60 min and then cooled. The reaction mixture was centrifuged at 4,000 rpm for 10 min after adding 1.0 ml of distilled water and 5.0 ml of *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). The levels of TBARS were expressed as the equivalents of malondialdehyde (MDA) amounts that were produced from 1,1,3,3-tetramethoxypropane.

Statistical analysis

All data are expressed as means with their standard deviations. The statistical significance of differences between the control and the OX-treated group was determined by the Student's *t* test or the Aspin-Welch's *t* test. A *p* value of less than 0.05 was regarded as statistically significant.

Results

Body and liver weights, and histopathological findings

Throughout the experimental period, OX treatment affected neither clinical symptoms nor food consumption. Body weight gains in OX-treated rats were suppressed from 2 to 3 weeks after partial hepatectomy; however the final body weight was not changed compared with that of animals in

the DEN alone group (data not shown). The absolute and relative liver weights were significantly increased in OX-treated rats compared with rats in the DEN alone group (Table 2). Histopathologically, OX induced hepatocellular hypertrophy and vacuolation of hepatocytes, which were the earliest signs of a compound-related effect in rats.

Table 2 Changes in parameters for body weight, liver weight, GST-P positive foci, oxidative damage and cell proliferation

Group	DEN + Basal diet	DEN + 0.05% OX
No. of rats examined	9	6
Final body weight (g)	253.3 ± 13.4	255.1 ± 14.4
Absolute liver weight (g)	6.2 ± 0.5	8.3 ± 0.6**
Relative liver weight (g/100 g body weight)	2.4 ± 0.1	33. ± 0.1**
GST-P positive foci (≥0.2 mm)		
Numbers (number/cm^2)	4.02 ± 1.94	12.74 ± 5.66*
Ares (mm^2/cm^2)	0.11 ± 0.07	0.21 ± 0.10*
8-OHdG (8-OHdG/10^5 dG)	0.16 ± 0.01	0.20 ± 0.02*
TBARS (nmol MDA/mg protein)	0.95 ± 0.06	1.14 ± 0.09*
PCNA-positive cells (%)	1.42 ± 0.32	4.18 ± 1.04**

Each quantitative data represents mean ± SD

*,** Significantly different from DEN control ($p < 0.05$, 0.01, respectively, Aspin–Welch's t test)

Effects of OX treatment on GST-P-positive foci and cell proliferation

The numbers and areas of GST-P-positive foci in the OX-treated group were significantly increased compared with those in the DEN alone group (Table 2). In addition, the effect of OX on cell proliferation was evaluated by immunohistochemistry for PCNA (Table 2). The number of PCNA-positive hepatocytes was significantly increased in OX-treated animals ($p < 0.01$).

Increased expression of genes encoding phase I and phase II drug-metabolizing enzymes

In order to evaluate the effect of OX on the hepatic expression of xenobiotic detoxification- and oxidative stress-related genes, qRT-PCR analysis was performed in the livers of five rats per group using the primers listed in Table 1. Significant increases in the expression levels of genes encoding phase I drug-metabolizing enzymes, such as *Cyp1a1*, *Cyp1a2* and *Nqo1*, were observed in animals in the OX-treated group (Fig. 1). OX also significantly induced the expression of NF-E2-related factor 2 (Nrf2)-regulated genes, such as *Gpx2*, *Yc2*, *Afar*, *Gstm1*, *Me1* (and also *Nqo1*), the products of which mainly functioned as phase II drug-metabolizing enzymes (Fig. 1).

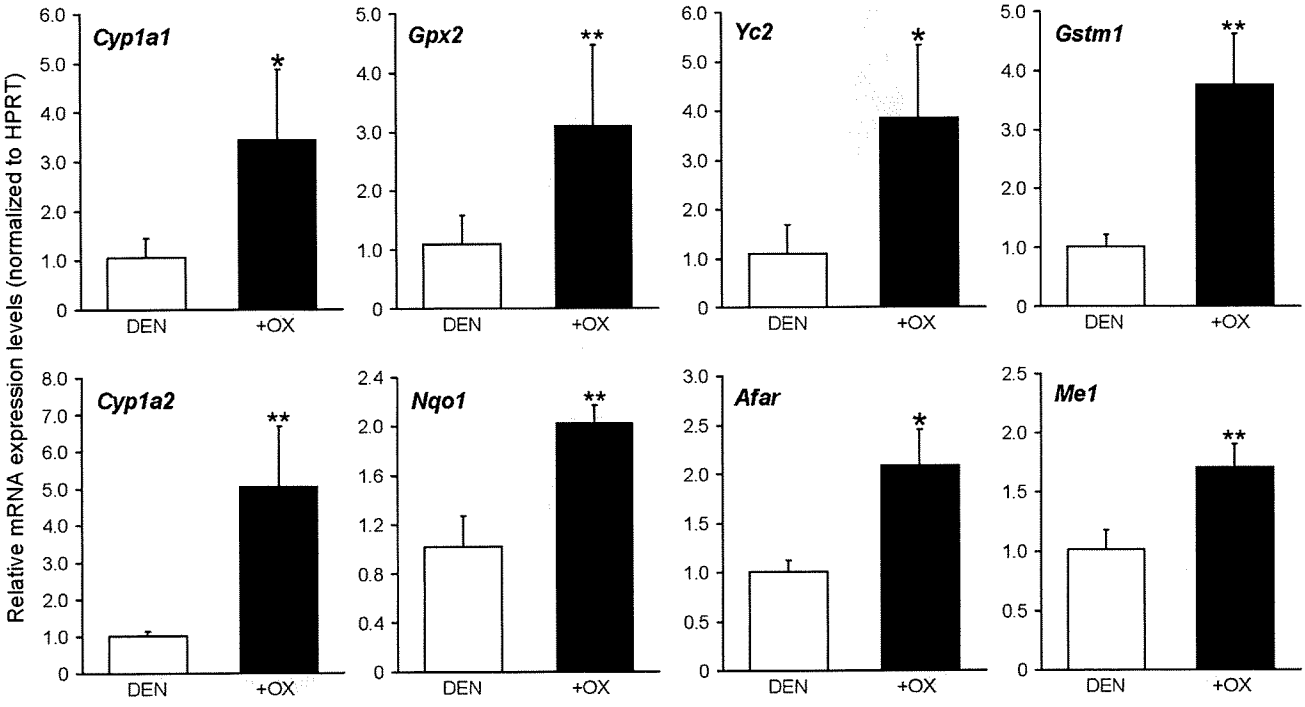

Fig. 1 Increased mRNA expression levels of phase I and phase II drug-metabolizing enzymes, and Nrf2-regulated molecules confirmed by quantitative real-time RT-PCR (qRT-PCR). Each column represents the mean + SD of the increased mRNA expression levels in the livers of 5 partially hepatectomized rats given 0% (*white column*) or 0.05% (*black column*) of OX after DEN initiation. *, ** indicates significant differences from DEN control ($p < 0.05$, 0.01 respectively; Aspin–Welch's t test)

Enhancement of microsomal ROS production

In order to estimate the cellular sources of ROS, NADPH-dependent ROS production was measured in isolated liver microsomes (Fig. 2a). Without NADPH, oxidised H_2DCFDA was not observed as an indicator of ROS production. However, ROS production was drastically enhanced by the addition of NADPH into the microsomal system, and its amount was statistically increased in rats given 0.05% OX compared with those in the DEN alone group. A well-known inhibitor of P450—SKF-525A—effectively inhibited these enhancements. Furthermore, the protein expression of CYP1A1 was concomitantly increased in microsomes isolated from the livers of rats treated with OX. On the other hand, the protein expression of CYP2E1, which was reported to predominantly generate ROS and to be related to oxidative stress (Gonzalez 2005), was unchanged by OX treatment (Fig. 2b).

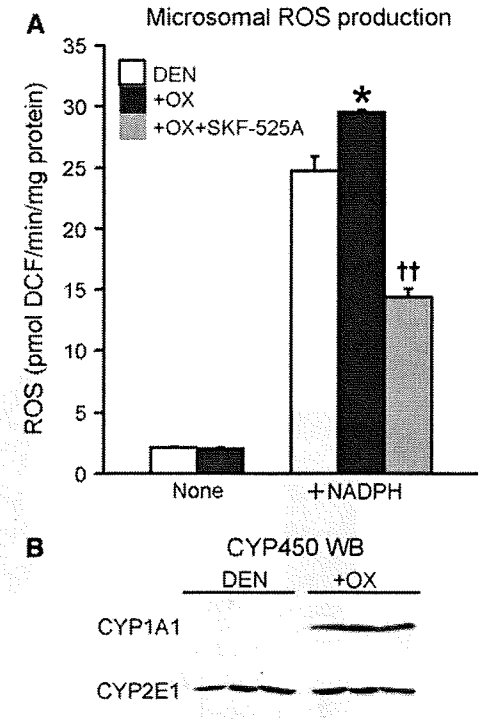

Fig. 2 Enhancement of ROS production and CYP1A1 protein expression in hepatic microsomes isolated from rats given OX. **a** NADPH-dependent microsomal ROS production was measured using the fluorescent probe H_2DCFDA. Each column represents the mean + SD values of ROS production in isolated microsomes from the livers of 9 or 6 partially hepatectomized rats given 0% (*white column*) or 0.05% of OX (*black column*) after DEN initiation. The *gray column* shows that the in vitro addition of SKF-525A (0.1 mmol/l) significantly suppresses ROS production in microsomes isolated from rats treated with 0.05% OX. *indicates significant differences from DEN control ($p < 0.05$; Aspin–Welch's t test). †† indicates significant suppression of ROS production by SKF-525A in rats treated with 0.01% OX ($p < 0.01$; Aspin-Welch's t test). **b** Western blot analysis for CYP1A1 and CYP2E1 protein expression for each microsome fraction used in (**a**)

Increase in oxidative stress changes

In order to evaluate whether oxidative damage to cellular components occurs during the formation of preneoplastic foci, 8-OHdG content and the level of TBARS formation were determined in livers (Table 2). The contents of 8-OHdG and TBARS were slightly but significantly increased in rats given 0.05% OX compared with those in the DEN alone group.

Discussion

OX, FEN, and their prodrug febantel (FB) have been widely utilized as anthelmintics in veterinary fields. FEN is metabolically interconvertible to OX, which is its most effective pharmacological form. FB is not a benzimidazole, but is converted in vivo by cyclization to FEN and subsequent oxidation at the sulphur atom to OX; thus, both FEN and FB are metabolized to OX. These three anthelmintics showed no apparent mutagenicity in numerous tests, including the Ames/Salmonella test, in vivo cytogenetics assays, and micronucleus assays (WHO 1991). With regard to their carcinogenicity, the 50th meeting of JECFA finally concluded that OX has no carcinogenic potential in rats and mice although OX might have tumor-promoting potential in rats; thus, the acceptable daily intake (ADI) of 0–7 μg/kg/day was set as a group ADI for OX, FEN and FB in JECFA (WHO 1999). However, the mechanisms underlying the tumor-promotion activity of OX in rats, at the molecular level, have still not been clarified.

Quantitative real-time PCR analysis revealed that phase I (*Cyp1a1*, *Cyp1a2*) drug-metabolizing enzymes were up-regulated in the livers of rats treated with OX. The induction of *Cyp1a1* and *Cyp1a2* observed in this study was in agreement with previous reports on several benzimidazole class compounds. OX induced CYP1A2 protein in the rabbit liver (Gleizes et al. 1991); FEN, albendazole and mebendazole induced CYP1A1 and CYP1A2 proteins in primary rat hepatocytes and HepG2 cells (Baliharova et al. 2003); thiabendazole, an anthelmintic and fungicide, induced *Cyp1a1* in rabbit hepatocytes in in vitro conditions (Aix et al. 1994); omeprazole, a gastric pump inhibitor, induced *Cyp1a1* and *Cyp1a2* in primary human hepatocytes (Diaz et al. 1990) and rat hepatocytes (Lemaire et al. 2004). The activation of *Cyp1a1* gene expression induced by omeprazole and thiabendazole does not require their binding to the aryl hydrocarbon receptor (Daujat et al. 1992; Aix et al. 1994), but it depends on a protein tyrosine kinase-mediated signal transduction pathway in HepG2 cells (Kikuchi et al. 1998) and rat hepatocytes (Lemaire et al. 2004). Indeed, benzimidazoles are atypical CYP1A inducers, which do not require their binding to AhR to induce CYP1A enzymes.

It has been reported that CYP1A1 induction is also related to the production of reactive oxygen species (ROS) induced not only by classical AhR ligands, such as TCDD (Park et al. 1996; Knerr et al. 2006) and coplanar polychlorinated biphenyl congeners (Schlezinger et al. 2006), but also the atypical, non-AhR ligand dicyclanil, which is an insecticide (Moto et al. 2005). Indeed, we have confirmed that microsomes isolated from the livers of rats treated with OX showed enhanced ROS production with a concomitant increase in CYP1A1 protein expression. It is generally accepted that microsomal CYP450s sequentially transfer 2 electrons to oxygen from microsomal NADPH-cytochrome P450 reductase 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 metabolised. Indeed, PCB increases CYP1A1-dependent microsomal ROS production in the livers of rats as well as scup (*Stenotomus chrysops*) (Schlezinger et al. 2006). Therefore, excessive amounts of OX can induce CYP1A enzymes and subsequently enhance microsomal ROS production. On the other hand, Shertzer et al. (2006) recently reported that TCDD decreased hepatic ATP levels and altered the mitochondrial integrity in mice, which contributed to generate an oxidative stress such as oxidative DNA damages. In the present study, we have not examined the effect of OX treatment on mitochondrial functions, and therefore it is necessary to perform further studies with regard to its tumor modifying effect as a future work.

In addition, we observed increased gene expression levels of Nrf2-regulated, anti-oxidative stress genes (*Gpx2*, *Nqo1*, *Yc2*, *Akr7a3*, *Gstm1* and *Me1*) by quantitative real-time RT-PCR. In these genes, Me1 is a NAD(P)H-regenerating enzyme, and its increased expression may be beneficial for the function of the detoxifying enzymes, directly (NQO1, Akr7a3), or indirectly (Gpx2, Yc2) (Thimmulappa et al. 2002). Collectively, these results suggest that OX triggers oxidative stress responses, and that the gene expression levels of phase I and phase II enzymes are intrinsically induced to maintain the cellular redox balance.

We also estimated whether resultant oxidative damage occurs in the cellular components during preneoplastic foci formation. The amount of 8-OHdG in the nuclear DNA of rats given OX was significantly higher than that in the nuclear DNA of rats in the DEN alone group. 8-OHdG adducts have been reported to cause misreading of the DNA sequence during replication, thereby inducing G:C to T:A transversion, which is involved in carcinogenesis (Cheng et al. 1992). In fact, some CYP1A inducers such as dicyclanil and piperonyl butoxide increase the hepatic 8-OHdG levels during their hepatocarcinogenesis in mice or rats (Moto et al. 2005; Muguruma et al. 2007), and therefore, it is speculated that increases of 8-OHdG attributed to OX treatment may contribute its hepatocellular tumor promoting activity. In addition, OX induced a slight but significant increase in the level of lipid peroxidation. A positive correlation between lipid peroxidation and the induction of preneoplastic lesions has been reported in the livers of rats treated with DEN followed by treatment with 2-acetylaminofluorene and partial hepatectomy (Sanchez-Perez et al. 2005). In addition, the cellular level of lipid peroxidation paralleled the degree of malignancy in a comparison between a baby hamster kidney cell line (BHK-21/C13) and its polyoma virus-transformed malignant counterpart (Goldring et al. 1993). Our data suggest that OX induces sustained oxidative stress in the livers of rats, which accounts for, at least in part, the enhancement in microsomal ROS production and an increased level of oxidative DNA damage as well as lipid peroxidation. Such oxidative stress responses overwhelming detoxifying systems might contribute to the tumor-promoting activity of OX.

In conclusion, we have demonstrated that OX exhibits tumor-promoting activity that enhances oxidative stress and preneoplastic foci in a DEN-initiated hepatocarcinogenesis model in partially hepatectomized rats. The prolongation of this tumor-promotion effect may induce hepatocellular tumors in rats if high doses of OX are administered for a long term.

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Original Article

Suppressive effect of *Siraitia grosvenorii* extract on dicyclanil-promoted hepatocellular proliferative lesions in male mice

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ABSTRACT — Dicyclanil (DC) generates reactive oxygen species (ROS) due to *Cyp1a1* induction, and DNA damage caused by oxidative stress is probably involved in hepatocarcinogenesis in mice. To clarify the modifying effect of the *Siraitia grosvenorii* extract (SGE), which has antioxidative properties, we employed a 2-stage liver carcinogenesis model in partially hepatectomized male ICR mice. Mice maintained on diet containing DC at a concentration of 1,500 ppm for 9 weeks after a single intraperitoneal injection of diethylnitrosamine (DEN) at a dose of 30 mg/kg and they were given water containing 2,500 ppm of SGE for 11 weeks including 2 weeks as pre-administration on DC. SGE inhibited the induction of γ-glutamyltranspeptidase-positive hepatocytes, lipid peroxidation, and gene expression of *Cyp1a1*, all of which were caused by DC. To examine whether SGE indirectly inhibits *Cyp1a1* expression induced by inhibition of aryl hydrocarbon receptor (Ahr)-mediated signal transduction caused by DC, mice with high (C57BL/6J mice) and low affinities (DBA/2J mice) to Ahr were given DC-containing diet and/or SGE-containing tap water for 2 weeks. *Cyp1a1* gene expression was significantly lower in C57BL/6J mice administered DC + SGE than in C57BL/6J mice administered DC alone; there was no difference in the *Cyp1a1* expression between DBA/2J mice administered DC + SGE and DC alone. These results suggest that SGE suppresses the induction of *Cyp1a1*, leading to inhibition of ROS generation and consequently inhibited hepatocarcinogenesis, probably due to suppression of Ahr activity.

Key words: *Siraitia grosvenorii* extract, Dicyclanil, Chemoprevention, Aryl hydrocarbon receptor, *Cyp1a1*, Oxidative stress

INTRODUCTION

Siraitia grosvenorii (*Cucurbitaceae*), a traditional medicinal herb grown in China, has been used as a folk medicine for treatment of lung congestion, cold, and sore throat. The major component of this plant extract is triterpene glycosides which are predominantly composed of mogrosides. These glycosides are 400 times sweeter than sucrose (Kasai *et al.*, 1998). In addition, since the triterpene glycoside in *Siraitia grosvenorii* contains sapogenin with a triterpenol structure and the glucosidic bond is β-bond-like fiber, it is not decomposed and digested by amylase in humans and cannot be much absorbed and converted into energy, thereby contributing to lower calories (Song *et al.*, 2007). The non-caloric sweetening property of *Siraitia grosvenorii* extract (SGE) makes it useful as a substitute for sugar to prevent obesity and diabetes. Recent researches on the triterpene glycoside contained in SGE have focused more on its *in vitro* and *in vivo* anticarcinogenic and antioxidative effects. For example, SGE reduces the atherogenic potential of low-density lipoprotein (LDL) by inhibition of copper-mediated oxidation and human umbilical vein endothelial cell-mediated oxidation (Takeo *et al.*, 2002). In particular, 11-oxo-mogroside V in SGE has been shown to inhibit the tumor-promoting activity of 12-*O*-tetradecanotiphorbol-13-acetate in a two-stage skin carcinogenesis model in mice and the tumor-initiating activity of peroxinitrite (Takasaki *et*

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al., 2003). Additionally, 11-oxo-mogroside V in SGE has been also reported to have inhibitory effects on reactive oxygen species (ROS) and DNA oxidative damage (Chen *et al.*, 2007). In an *in vivo* experiment, the development of diabetic nephropathy was prevented in diabetic mice treated with SGE due to its antioxidative action (Song *et al.*, 2007). However, the precise mechanisms underlying physiological and pharmacological properties responsible for the antioxidative and anticarcinogenic effects of SGE remain unclear.

Dicyclanil (DC), 4,6-diamino-2-cyclopropylamino-pyrimidine-5-carbonitrile, is a pyrimidine-derived insect growth regulator that inhibits molting and development of insects and is used in the field of veterinary medicine to prevent myiasis in sheep. It has been evaluated in the 54th meeting of the Joint Food and Agriculture Organization (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA) that DC is a non-genotoxic hepato-carcinogen (WHO 2000). In our previous study using a two-stage hepatocarcinogenesis model in which male mice were fed a diet containing DC at a concentration of 1,500 ppm for 13 or 26 weeks after partial hepatectomy and dimethylnitrosamine (DMN) initiation treatment, the number and area of γ-glutamyltranspeptidase (GGT)-positive foci, a marker enzyme for preneoplastic hepatocytes in the liver (Carter *et al.*, 1985; Cameron *et al.*, 1978), was found to increase significantly in the DMN + DC group as compared to that in the DMN group (Moto *et al.*, 2006a). Significant increases in mRNA expressions of some metabolism- and oxidative stress-related genes such as *Cyp1a1*, *Por*, *Sod1*, *Txnrd1*, and *Ogg1* were also observed in the DMN + DC group. The *in vitro* measurement of ROS generation from mouse liver microsomes revealed a significant increase in ROS production in the presence of DC. These results suggest that DC induces oxidative stress, which is probably derived from its metabolic pathway, and plays an important role in DC-induced hepatocarcinogenesis in male mice.

In the present study, to investigate the antioxidative properties of SGE, we examined the modifying effect of SGE on liver tumor-promoting effect of DC in male ICR mice and investigated the possible molecular mechanism of the tumor-modifying effect of SGE.

MATERIALS AND METHODS

Animals and chemicals

In the present study, we performed 2 experiments. We purchased 5-week-old male ICR mice for Experiment 1 and 5-week-old male C57BL/6J and DBA2 mice for Experiment 2 from Japan SLC Inc. (Shizuoka, Japan).

All animals were maintained on a powdered basal diet and tap water during the 1-week accommodation period. During the course of the experiment, they were maintained under the conventional conditions (room temperature, 22 ± 2°C; light/dark cycle, 12 hr). Their body weights and food and water consumptions were measured once a week. The experiment was carried out in accordance with the Guide for the Animal Experimentation of the Tokyo University of Agriculture and Technology.

SGE was kindly provided by Saraya Co., Ltd. (Osaka, Japan). In extraction procedure, fresh *Siraitia grosvenorii* was washed and crushed; then, anextraction was done using hot water at a temperature range of 80-90°C. After filtration, the extract obtained was evaporated under reduced pressure. The moisture content of the extract was about 40 w/w%. To prepare the *Siraitia grosvenorii* glycoside, *Siraitia grosvenorii* extract in paste form was diluted and the sweet components were selectively adsorbed onto a reversal phase column. Using an ethanolic solution, the sweet components that had been adsorbed onto the columns were eluted. Ethanol was then removed. Afterwards, spray drying was used to change the *Siraitia grosvenorii* glycosides from a liquid state into powder form. The concentration of mogroside V was about 31 w%. (Hossen *et al.*, 2005) As a preliminary study to select the appropriate dose of SGE, male ICR mice (5 animals per group) were fed a diet containing 0, 25, 250 or 2,500 ppm SGE for 4 weeks. As a result, SGE showed a dose-dependent decrease in *Cyp1a1* expression, and the decreasing expression was marked in the 2,500 ppm group. From these results, the dose of 2,500 ppm SGE was decided to be appropriate in Experiment 1.

DC was kindly provided by Novartis Animal Health Inc. (Basel, Switzerland), and *N*-diethylnitrosamine (DEN) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

Experiment 1

Experimental design

A two-stage hepatocarcinogenesis model in mice was employed. Total of 24 mice was divided into the following 3 groups, each comprising 8 animals: DEN alone (gr. I), DEN + DC (gr. II), and DEN + DC + SGE (gr. III). To enhance the hepatocellular proliferation, all animals in grs. I to III were subjected to two-thirds partial hepatectomy, and after 12 hr, they were administered a single intraperitoneal injection of DEN at a dose of 30 mg/kg. Pretreatment with triterpene has been reported to alter the redox system of tissues by scavenging the free radicals

and improving the antioxidant status of the liver (Sunitha *et al.*, 2001); hence, the animals in gr. III were given tap water containing 2,500 ppm SGE 1 week before initiation treatment, while those in grs. I and II were given tap water alone. One week after the initiation treatment, animals in grs. II and III were fed a diet containing DC at a concentration of 1,500 ppm until 9 weeks (Fig. 1). Three mice died due to the hepatectomy and initiation treatment. For liver sampling at 11 weeks, the mice were sacrificed by exsanguination of the posterior vena cava under ether anesthesia after measuring their body weights. The livers were excised, macroscopically examined, weighed, and cut into sections. For all mice, one liver section was fixed with 4% paraformaldehyde for 24 hr for histological and immunohistochemical examinations. Another liver section was embedded in an optimal cutting temperature (OCT) compound (Tissue-Tek; Sakura Finetek Japan Co., Tokyo, Japan) before freezing for evaluation of GGT-positive cells, a marker of preneoplastic foci in mouse liver. The remaining liver samples were stored at −80°C for subsequent gene and protein expression analyses.

Histological and histochemical examinations

For histological examinations, liver tissues were fixed with 4% paraformaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). The histochemical staining of GGT was performed using the method proposed by Rutenberg *et al.* (1969) with some modifications. The frozen tissues were sectioned with a cryotome and fixed with methanol. After air drying, the tissue sections were coated with freshly prepared solution containing the substrate L-glutamic acid-γ-(4-methoxy-β-naphthylamide) (Sigma-Aldrich, St. Louis, MO, USA) and fast blue BBN (Wako Pure Chemical Industries, Japan) in 0.1 M Tris-buffered saline (pH 7.4). Following incubation, the slides were transferred into a 0.1 M cupric sulfate solution. The thin sections were then stained with hematoxylin and mounted in Apathy's mounting medium. On GGT evaluation, the number of positive cells per area was calculated from the total area of the tissue sections by using a computer-assisted image analyzer (NIH image).

Immunohistochemical examinations

Cell proliferation was assessed by immunohistochemical staining for proliferating cell nuclear antigen (PCNA). Thin sections of paraffin-embedded tissues (3 μm) were incubated with monoclonal anti-PCNA antibody (1 : 50; PC10, DAKO, Glostrup, Denmark), followed by Histofine Simple Stain Mouse MAX PO (M) procedures (Universal Immunoperoxidase Polymer for staining mouse tissue sections; Nichirei, Tokyo, Japan). Hydrogen peroxide with a coloring agent diaminobenzidine (DAB) was used as the substrate. The number of PCNA-positive cells per 2,000-3,000 cells on each slide was counted from 10 different areas.

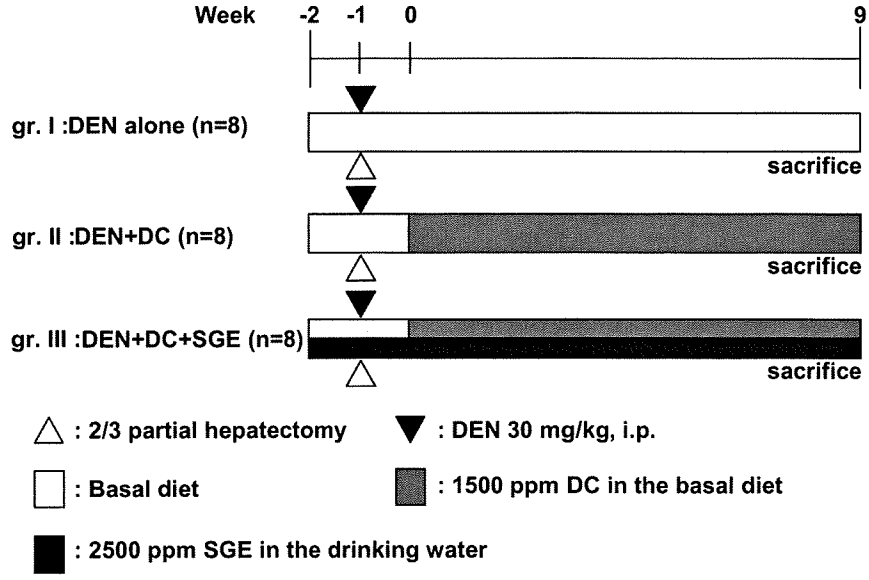

Fig. 1. Experimental design in Experiment 1.

cDNA microarray analysis

The gene expression in the liver samples obtained from mice in groups II and III was analyzed using 2 types of low-density pathway-specific microarrays (Mouse Stress and Toxicity Pathway Finder Gene Array and Mouse Drug Metabolism Gene Array: GEArray; SuperArray Bioscience, Frederick, MD, USA). A list of all genes in this microarray is available on the web site (http://www.superarray.com/gene_array_product/HTML/MM-012.html). Liver samples were selected from one mouse in each group. Total RNA was extracted using TRIzol (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's instructions. The cDNA was synthesized from 3 μg of total RNA by using the AmpoLabeling-LPR Kit (SuperArray Bioscience Corp.) with the conversion of total RNA biotinylated cDNA probes. The array membranes were hybridized overnight with biotin-labeled probes at 60°C. The filters were then washed twice with 2 × saline sodium citrate buffer (SSC)/1% sodium dodecyl sulfate (SDS), followed by washing twice with 0.1 × SSC/1% SDS at 60°C for 15 min each. Chemiluminescence was detected by subsequent incubation of the filters with alkaline phosphatase-conjugated streptavidin and CDP-Star substrate, followed by exposure to Hyperfilm™-ECL X-ray film (Amersham Biosciences UK Ltd., Buckinghamshire, UK). The image data obtained from GEArray were analyzed using the GEArray Expression Analysis Suite software (http://www.geasuite.superarray.com/index.jsp); correction for background noise was performed by subtracting the minimum value and normalizing to the value of the housekeeping genes (β-actin). For each spot, the ratio of intensities between grs. II and III was analyzed. The genes in gr. III were considered significant if the value of changes was less than 0.5-fold or greater than 2.0-fold as compared to that in gr. II. Based on the results of the cDNA microarray analyses in the present study and those of our previous study regarding the DC-induced hepatocarcinogenesis (Moto *et al.*, 2006b), the genes involved in detoxification and oxidative stress were selected for the analyses of quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR).

Real-time RT-PCR

Quantitative real-time RT-PCR was carried out to validate the genes listed in Table 1. Briefly, total RNA from 4 animals, including the mice used for cDNA microarray analysis in each group, was extracted using TRIzol (Invitrogen Corp.), according to the manufacturer's instructions. After measuring the total RNA concentration using an electrophotometer and determining the RNA quality by spectrometry, cDNA was synthesized from 2 μg of RNA in the presence of dithiothreitol (DTT), dNTPs, random primers, RNaseOUT (Invitrogen Corp.), and SuperScript™ III Reverse Transcriptase (Invitrogen Corp.) in a 20 μl total reaction mixture. Quantitative real-time RT-PCR with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) was performed using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The real-time RT-PCR reaction was performed according to the SYBR Green PCR Master Mix protocol. The PCR primers were designed using the Primer Express software (Applied Biosystems). The amount of target genes, normalized to an endogenous control (β-actin) and relative to a control, was determined by the 2_ΔΔCt method (Livak and Schmittgen, 2001).

Lipid peroxidation

Lipid peroxidation was measured by the formation of thiobarbituric acid-reactive substances (TBARS) (Ohkawa *et al.*, 1979). Liver tissues (1.5-2.0 g) stored at −80°C were homogenized in 1.15% KCl on ice. Liver homogenate (30%; 0.1 ml) was mixed with 8.1% SDS, 20% acetate buffer solution, 0.8% 2.6-di-*t*-butyl-4-methylphenol (BHT), and 0.8% thiobarbituric acid (TBA); the reaction mixture was incubated at 95°C for 30 min, and the reaction was terminated by placing samples under cold water. Centrifugation was conducted at 3,000 rpm for 10 min after adding 0.5 ml distilled water and 2.5 ml *n*-butanol and pyridine (15 : 1 v/v). Absorbance of the resulting solution in *n*-butanol phase was measured spectrophotometrically at a wavelength of 532 nm by using the Synergy HT Multi-Detection Microplate Reader (BioTek). Malondialdehyde (MDA), obtained by acid hydrolysis of 1,1,3,3-tetraethoxypropane (TEP), was used as the standard for the quantification of TBARS. Data was expressed as nmol of MDA per gram of the liver.

Experiment 2

To examine whether SGE indirectly inhibits CYP1A1 expression by inhibiting aryl hydrocarbon receptor (Ahr)-mediated signal transduction caused by DC, the following experiment was carried out using genetically defined mice with high affinity (C57BL/6J mice) and low affinity (DBA/2J mice) to Ahr. Compared to the C57BL/6J mice, the DBA/2J mice require a 10-20 times higher 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) dose to manifest toxicity (Chapman *et al.*, 1985). The combination of these animal models has been used in typical CYP1A inducers, including benz[a]anthracene, β-naphthoflavone, and TCDD, in order to examine the effects of these chemi-

Table 1. Sequences of primers used in the real-time RT-PCR analysis

Symbol	Gene Name	Forward primer	Reverse primer	Accession No.
Cyp1a1	Cytochrome P450, family 1, subfamily a, polypeptide 1	AGGATGTGTCTGGTTACTTTG	AGAAACATGGACATGCAAG	NM_009992
Cyp1a2	Cytochrome P450, family 1, subfamily a, polypeptide 2	GCTACTTGTGCATGGCCTA	AAGCCATTCAGTGAGGTGTC	NM_009993
Cyp2a5	Cytochrome P450, family 2, subfamily a, polypeptide 5	GCCAACGTTATGGTCCTGTATTC	GTCCGCACAGCACCACAA	NM_007812
Cyp2e1	Cytochrome P450, family 2, subfamily e, polypeptide 1	TCAAAAAGACCAAAGGCCAGC	TCCGCAATGACATTGCAGG	NM_021282
Cyp7a1	Cytochrome P450, family 7, subfamily a, polypeptide 1	AAGCACAGATTCTCCCCTTGG	CAGCAATCCCCCAGATCAAA	NM_007824
Aldh1a1	Aldehyde dehydrogenase family 1, subfamily A1	GACTTGAAGATTCAACATACC	TCACAGCTTTGTCAACATCA	NM_013467
Gstμ2	Glutathionr S-transferase mu 2	TTCCCAATCTGCCCTACTTGA	TCTCCACACAGGTTGTGCTTTC	NM_008182
Gstα2	Glutathionr S-transferase alpha 2	ACATGAAGGAGAGAGCCCTGAT	GCAGTCTTGGCTTCTCTTTGGT	NM_008183
Nrf2	NF-E2 related factor 2	CGACAGAAACCTCCATCTACTGAA	CCTCATCACGTAACATGCTGAAG	NM_010902
HO-1	Heme oxigenase 1	TCGTGCTCGAATGAACACTCTG	AAGGCGGTCTTAGCCTCTTCTG	NM_010442
por	Cytochrome P450 oxidoreductase	GCCTGCCTGAGATCGACAAG	GGGTCGCCTTCTCCGTATGT	NM_008898
Gpx2	Glutathione peroxidase2	GCTGCCCTACCCTTATGATGAC	CGCACGGGACTCCATATGAT	NM_030677
Ahr	Aryl hydrocarbon receptor	CGCTGAAACATGAGCAAATTGG	ACAGCTTAGGTGCTGAGTCACGG	NM_01364
Arnt	Aryl hydrocarbon receptor nuclear translocator	GATGCGATGATGACCAGATGTG	CAGTGAGGAAAGATGGCTTGTAGG	NM_009709
βactin	beta actin	AGATTACTGCTCTGGCTCCTAGCA	GCCACCGATCCACACAGATG	NM_007393

cals on the expressions of cyp1A1 and Ahr (Prochaska and Talalay, 1998).

A total of 20 male mice, including C57BL/6J and DBA/2J mice, were divided into the following 6 groups, each comprising 3 or 4 animals: untreated (grs. IV and VII of C57BL/6J and DBA/2J mice, respectively), DC alone (grs. V and VIII of C57BL/6J and DBA/2J mice, respectively), and DC + 2,500 ppm SGE (grs. VI and IX of C57BL/6J and DBA/2J mice, respectively) groups. The mice in grs. IV and VII were fed powdered basal diets and tap water for 3 weeks. The mice in grs V, VI, VIII, and IX were fed powdered basal diets for the first week, and a diet containing 1,500 ppm DC for the following 2 weeks. The mice in grs. VI and IX were given tap water containing 2,500 ppm SGE for 3 weeks, including 1 week of pretreatment period, while the mice in grs. V and VIII were given tap water for 3 weeks (Fig. 3A). All surviving animals were sacrificed by exsanguination of the posterior vena cava under ether anesthesia after measuring the body weights, and the liver samples were frozen in liquid and stored at −80°C. The quantitative real-time RT-PCR was carried out to measure the gene expression of *Cyp1a1*.

Statistical evaluation

The data obtained by measuring body and liver weights, GGT-positive and PCNA-positive cells, TBARS, and real-time RT-PCR analysis were expressed as mean ± S.D.. We compared the difference between grs. I, IV, VII and II, III, V, VI, VIII, IX and that between grs. II, V, VIII and III, VI, IX by using Student's *t* test. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Experiment 1

There was no remarkable difference in the final body weight among the three groups. The relative weights of the liver significantly increased in grs. II and III compared with those in gr. I (Table 2). The relative weight of the liver in gr. III was not significantly different from that in gr. II.

Histopathologically, hypertrophy of centrilobular hepatocytes with vacuolation was observed in mice from grs. II and III. Degenerative lesions and single cell necroses were also found in these groups. The severities of these changes in grs. II and III were almost the same.

On histochemical staining of GGT, GGT-positive reaction was observed as a single cell but not as a focus. In grs. II and III, the number of GGT-positive hepatocytes significantly increased compared with that in gr. I. On the contrary, this ratio in gr. III was significantly lower than that in gr. II (Table 2).

With regard to the immunohistochemistry of PCNA, a significant increase in the ratio of PCNA-positive hepatocytes was observed in grs. II and III as compared to gr. I. This mean ratio in gr. III showed a slight but not significant decrease as compared to that in gr. II (Table 2).

The microarray analysis of the liver of mice from gr. III showed that 23 out of 96 genes in the pathway of Mouse Stress and Toxicity and 25 out of 96 genes in the pathway of Drug Metabolism were over- or underexpressed as compared to gr. II (Data not shown). Among them, 19 out of 23 genes in the pathway of Mouse Stress and Toxicity and 22 out of 25 genes in the pathway of Drug Metabolism were underexpressed by less than 0.5-fold. To clarify the mechanism of DC-induced hepatocellular tumors in mice based on the data of the present microarray analysis (underexpressed 19 genes in the pathway of Mouse Stress and Toxicity and 22 in the pathway of Drug Metabolism) and those of our previous studies, we selected 14 target genes that were normalized to β-actin as the internal control for the real-time RT-PCR analysis (Table 1). The changes in the selected genes observed by real-time RT-

Table 2. Body and liver weights, PCNA positive rations, GGT positive cells, and TBARS in Experiment 1

Group	gr. I	gr. II	gr. III
Body weight (g)	43.4 ± 2.1	41.9 ± 3.6	40.2 ± 2.2
Liver weight (g)	2.2 ± 0.1	2.6 ± 0.2$^{\#}$	2.3 ± 0.3
Relative liver weight (%)	5.0 ± 0.4	6.5 ± 0.3$^{\#}$	5.8 ± 0.5$^{\#}$
PCNA labeling index (%)	5.6 ± 1.1	19.6 ± 1.1$^{\#\#}$	16.5 ± 2.6$^{\#\#}$
GGT positive cell (number/cm^2)	20.6 ± 11.4	235.2 ± 26.4$^{\#\#}$	157.1 ± 26.6$^{\#\#,*}$
TBARS (nmol MDA/g Liver)	1045.6 ± 104.3	1354.6 ± 89.0$^{\#\#}$	1102.9 ± 129.2$^{\#,*}$

#, ##; significantly different from the gr. I at $p < 0.05$, 0.01, respectively (Student's t-test).
*; significantly different from the gr. II at $p < 0.05$ (Student's t-test). Data show mean ± S.D. values.

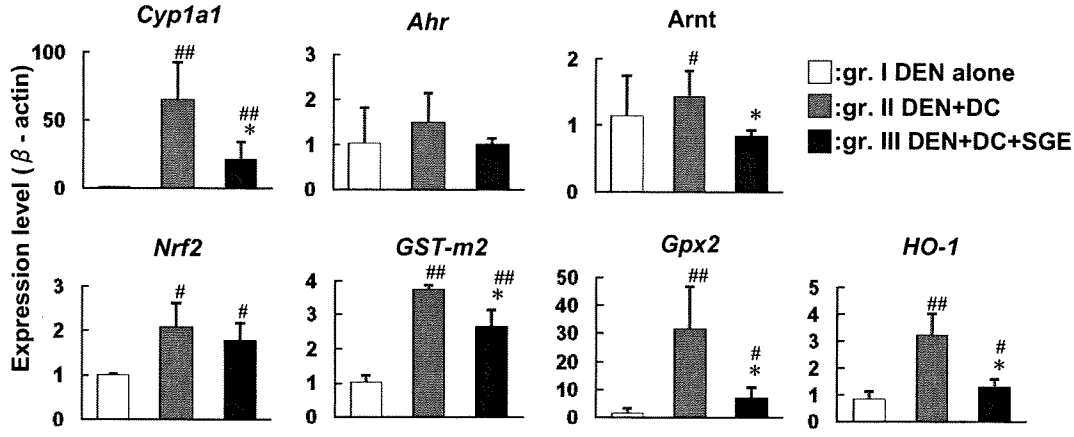

Fig. 2. mRNA expression analysis by real-time RT-PCR of the livers from mice in the grs. I (open column), II (grey column), III (black column) in Experiment 1. Individual gene expression levels were normalized using β-actin. Bars represent the mean ± S.D. of 4 mice. #, ## ; represents significantly different from gr. I at $p < 0.05$, 0.01, * ; represents significantly different from gr. II at $p < 0.05$ (Student's *t*-test).

PCR are shown in Fig. 2. The gene expressions of phase I enzymes such as *Cyp1a1*, significantly decreased in gr. III as compared to gr. II. In addition, the gene expressions of phase II enzymes such as *Gstm2*, *HO-1*, and *Gpx-2* significantly decreased in gr. III as compared to gr. II. There were no remarkable fluctuations in the gene expression of *Nrf2* and *Ahr* between grs. II and III. The results of real-time RT-PCR were consistent with those in the microarray analysis.

TBARS, an *in vivo* oxidative stress marker, significantly increased in grs. II and III compared to gr. I, but the value of gr. III was significantly lower than that in gr. II (Table 2).

Experiment 2

The gene expression of *Cyp1a1* significantly increased in gr. V (DC treated C57BL/6J mice) and gr. VIII (DC treated DBA/2J mice) as compared with that in the corresponding untreated groups (grs. IV and VII). The gene expression level of *Cyp1a1* in gr. VI (DC + SGE treated C57BL/6J mice) was significantly lower than that in gr. V (DC-treated C57BL/6J mice); however, there was no difference in the expressions between gr. VIII (DC treated DBA/2J mice) and gr. IX (DC+SGE treated DBA/2J mice) (Fig. 3B).

DISCUSSION

In Experiment 1, the administration of SGE to the DC-treated ICR mice subjected to DEN initiation and partial hepatectomy significantly decreased the *Cyp1a1* expression levels. The cytochrome P450 superfamily, which is responsible for the development of subsequent toxicity (Bock, 1994; Hankinson, 1985; Silvergeld *et al.*, 1989), is generally known to generate ROS as a byproduct of microsomal oxidation. *Cyp1a1*, a member of this family, has been reported to be the most active CYP enzyme for catalyzing procarcinogens (Guengerich and Shimada, 1991; Puntarulo and Cederbaum, 1998). In particular, the induction of *Cyp1a1* results in excessive generation of ROS due to the depletion of cellular antioxidants (Morehouse *et al.*, 1984; Stohs *et al.*, 1990). Therefore, the upregulation of *Cyp1a1* by the DC treatment could have increased the generation of ROS as a byproduct of microsomal oxidation. In the present study, we observed that SGE suppressed the induction of *Cyp1a1* and speculated that SGE indirectly decreased the amount of ROS generated by the metabolic pathway of DC. Significant decreases in the gene expression of phase II enzymes, antioxidant enzymes, and the level of TBARS that is a marker for lipid peroxidation (Carvalho *et al.*, 2007) were observed in gr. III of Experiment 1, indicating a decline in the amount of ROS generated. Furthermore, we observed that the number of GGT-positive cells, a marker of preneoplastic hepatocytes, decreased in gr. III as compared to gr. II. In our previous study of the mouse two-stage hepatocarcinogenesis model in DC, GGT-positive reaction was observed as a single cell but not as a focus in DC-treated groups in a short-term (13 weeks) study, while a significant increase in the number and area of GGT-positive foci was found in these groups in a long-term (26 weeks) study (Moto *et al.*, 2006a). This finding may suggest that

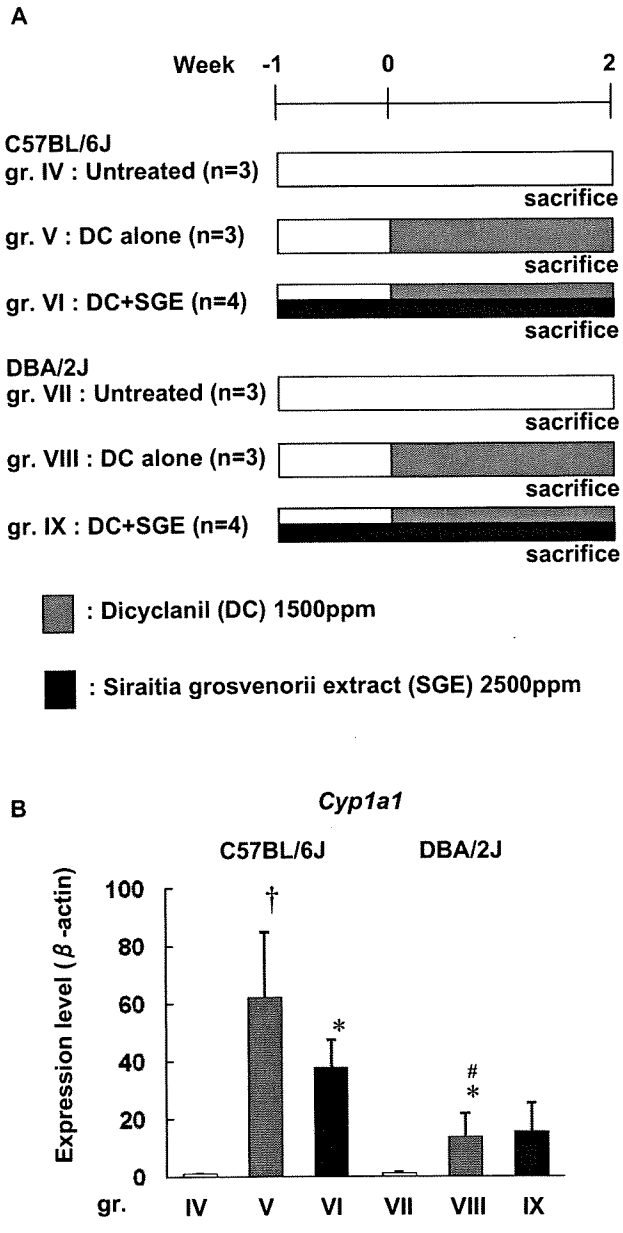

Fig. 3. **A.** Experimental design in Experiment 2.
B. mRNA expression of *Cyp1a1* of the livers from mice in grs. IV, VII (open column), V, VIII (grey column), VI, IX (black column). The gene expression levels was normalized using β-actin. Bars represent the mean ± S.D. of 3 mice. *; significantly different from gr. V at $p < 0.05$ respectively (Student's *t*-test). †; significantly different from gr. IV at $p < 0.01$ (Student's *t*-test). #; significantly different from gr. VII at $p < 0.01$ (Student's *t*-test).

GGT-positive cells, like GGT-positive foci, probably have a potential to function as a marker for preneoplastic hepatocytes in the liver. Based on the results of the present study and our previous studies, it can be considered that the development of preneoplastic cells promoted by DC after DEN initiation was suppressed by the SGE-induced inhibition of ROS generation.

Nrf2 plays a pivotal role in inducing the expression of the phase II detoxifying enzyme and the antioxidant protein that responds to oxidative stress (Itoh *et al.*, 2003). In the present study, the gene expressions of Gstμ2, a phase II enzyme; Gpx2, an antioxidant enzyme; and HO-1, a potent oxidative stress-responsive protein, significantly decreased in gr. III as compared to gr. II. The inducible expression of these genes depends on Nrf2 (Chanas *et al.*, 2002; Banning *et al.*, 2005; Cho *et al.*, 2006). DC-induced upregulations of the gene expression of phase I and II enzymes with a significant increase in the gene expression of Nrf2, while SGE significantly downregulated these gene expressions, along with a decrease in the gene expression of *Nrf2*.

The findings of Experiment 1 indicated that SGE suppressed the induction of *Cyp1a1*, and it might be an important part of the chemopreventive activity of SGE *in vivo*. The induction of *Cyp1a1* has been reported to depend on Ahr, which is a ligand-activated transcription factor in most cell and tissue types (Denison and Heath-Pagliuso, 1998). The interaction of the transformed Ahr complex with a specific dioxin responsive element of the nuclear DNA elicits the activation of certain genes, including *Cyp1a1*, in the cytochrome P450 superfamily (Neuhold *et al.*, 1989). The *Cyp1a1* induction has also been linked to the generation of ROS in not only the classical Ahr ligand, such as TCDD (Park *et al.*, 1996; Knerr *et al.*, 2006) and coplanar polychlorinated biphenyl congeners (Schlezinger *et al.*, 2006), but also the non-Ahr ligand. It is not clear whether DC induces *Cyp1a1* in association with Ahr. With regard to SGE, there is a report on the tumor-protective effect resulting from the function of antioxidant activities, but the mechanism underlying this effect has not been completely elucidated. Other natural antioxidants such as flavonoids (Fukuda *et al.*, 2007), catechins (Palermo *et al.*, 2003), and galangin (Hung *et al.*, 2006) have also been reported to suppress the Ahr transformation and downstream expression of *Cyp1a1*. In addition, resveratrol (Ciolino and Yeh, 1999), curcumin (Ciolino *et al.*, 1998), and certain vegetable constituents (Amakura *et al.*, 2003) act as antagonists of Ahr; this property is responsible for the inhibitory effects of these agents against the Ahr-induced carcinogenesis. Anti-oxidative properties include two mechanisms; directly scav-

enging ROS and indirectly suppressing ROS generation by inhibiting Ahr expression and above-referenced natural antioxidants, flavonoids, curcumin, have been reported to have both anti-oxidative properties (Kandaswami and Middleton, 1994; Fukuda *et al.*, 2007). The results of our study strongly suggest that SGE has a latter anti-oxidative property.

Thus, we performed Experiment 2 to examine the effect of SGE on Ahr and *Cyp1a1* expression induced by DC in genetically defined mice with high affinity (C57BL/6J mice) or low affinity (DBA/2J mice) to Ahr (Prochaska *et al.*, 1998). The aim of this experiment was to examine whether SGE indirectly inhibits *Cyp1a1* expression due to the DC-induced inhibition of Ahr-mediated signal transduction. In Experiment 2, the gene expression of *Cyp1a1* in both C57BL/6J and DBA/2J significantly increased in DC-treated groups (grs. V and VIII) as compared with that in the corresponding untreated groups (grs. IV and VII). However, the gene expression of *Cyp1a1* was significantly decreased in gr. VIII (DC treated DBA/2J mice) as compared to gr. V (DC treated C57BL/6J mice). This suggests the possibility that the induction of *Cyp1a1* in the metabolic pathway on DC may depend on Ahr. This is the first report to demonstrate that DC can induce the expression of *Cyp1a1* gene in an Ahr-dependent manner. In addition, the fact that the gene expression of *Cyp1a1* in group VI (DC + SGE treated C57BL/6J mice) significantly declined as compared to that in gr. V (DC treated C57BL/6J mice), while there were no significant differences in *Cyp1a1* between grs. VIII (DC treated DBA/2J mice) and IX (DC + SGE treated DBA/2J mice), indicates that SGE inhibits Ahr-induced *Cyp1a1* activity and suggests that SGE has antagonistic effects against Ahr. However, regarding the antagonistic effect of SGE on Ahr, the underlying molecular mechanism of action of SGE on Ahr has not been elucidated.

In conclusion, the results of our study strongly suggest the possibility that SGE plays an important role in suppressing the DC-induced generation of ROS in the metabolic process. This mechanism of the generation of ROS is probably derived from the activation of Ahr involving *Cyp1a1*. SGE might act on a certain component of this mechanism, leading to the suppression of the Ahr activity and downstream expression of *Cyp1a1*. The decreased incidence of GGT-positive cells in gr. III in Experiment 1 clearly suggests that SGE inhibits DC-induced hepatocarcinogenesis in a long-term experiment of mice initiated with DEN. SGE is used as a food item and a natural medicine in humans. Because of its low cost, proven chemopreventive potential, and pharmacological safety (Jin *et al.*, 2007), daily intake of SGE may possibly facilitate the maintenance of its constant blood concentration and prevent the generation of ROS. Due to a complex chemical structure of triterpene which is one of the main ingredients of SGE, digestive absorption of SGE and its subsequent metabolic pathway in the blood have not been determined. Though *in vitro* studies have demonstrated that the 11-oxo function of the B ring in the 11-oxo-mogroside V structure may be relevant with regard to its antioxidant activity (Chen *et al.*, 2007), extensive research is required to clarify the bioactive elements and the structure-effect relationship responsible for the antioxidant effects of SGE.

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GENOTOXICITY AND CARCINOGENICITY

Antioxidant enzymatically modified isoquercitrin or melatonin supplementation reduces oxidative stress-mediated hepatocellular tumor promotion of oxfendazole in rats

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Abstract To clarify whether enzymatically modified isoquercitrin (EMIQ) or melatonin (MLT) supplementation reduces oxidative stress-mediated hepatocellular tumor-promoting effect of oxfendazole (OX), a benzimidazole anthelmintic, male rats were administered a single intraperitoneal injection of *N*-diethylnitrosamine (DEN) and were fed a diet containing OX (500 ppm) for 10 weeks with or without EMIQ (2,000 ppm) or MLT (100 ppm) in the drinking water after DEN initiation. One week after the commencement of the administration of OX, rats were subjected to two-thirds of partial hepatectomy. The number of GST-P-positive foci promoted by OX was significantly inhibited by the combined antioxidant EMIQ or MLT administration, and the area of GST-P-positive foci was inhibited by the administration of MLT. Real-time RT–PCR analysis revealed decreases in mRNA expression levels of cytochrome P450, family 2, subfamily b, polypeptide 2 (*Cyp2b2*) and malic enzyme 1 (*Me1*) in the DEN-OX-EMIQ and DEN-OX-MLT groups and decreases in mRNA expression levels of *Cyp1a1* and aldo–keto reductase family 7, member A3 (*Akr7a3*) in the DEN-OX-MLT group compared to those in the DEN-OX group. In in vitro ROS production assay, inhibited production of NADPH-dependent ROS was observed by the treatment with EMIQ or MLT. These results suggest that coadministration of EMIQ or MLT suppresses the hepatocellular tumor-promoting activity of OX in rats through the decrease in ROS production by the activation of CYPs.

Keywords Oxfendazole · Tumor promotion · Rat · Antioxidant · Melatonin · Enzymatically modified isoquercitrin

Abbreviations

OX	Oxfendazole
EMIQ	Enzymatically modified isoquercitrin
MLT	Melatonin
AhR	Aryl hydrocarbon receptor
DEN	*N*-diethylnitrosamine
GST-P	Glutathione *S*-transferase placental form
Real-time RT–PCR	Quantitative real-time reverse transcription-polymerase chain reaction
Nrf2	NF-E2-related factor 2
ROS	Reactive oxygen species
H_2DCFDA	2′,7′-Dichlorodihydrofluorescein diacetate
DCF	2′,7′-Dichlorofluorescein
NADPH	Nicotinamide adenine dinucleotide phosphate
Cyp1a1	Cytochrome P450, family 1, subfamily a, polypeptide 1

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Cyp1a2	Cytochrome P450, family 1, subfamily a, polypeptide 2
Cyp2b2	Cytochrome P450, family 2, subfamily b, polypeptide 2
Nqo1	NAD(P)H dehydrogenase, quinone 1
Gstm1	Glutathione *S*-transferase, mu 1
Gpx2	Glutathione peroxidase 2
Akr7a3	Aldo–keto reductase family 7, member A3
Yc2	Glutathione *S*-transferase Yc2 subunit
Me1	Malic enzyme 1
AFAR	Aflatoxin B1 aldehyde reductase

Introduction

Enzymatically modified isoquercitrin (EMIQ) is a mixture of quercetin glycoside, which consists of isoquercitrin (IQ) and its α-glucosylated derivatives, with 1–7 additional linear glucose moieties (Akiyama et al. 2000). EMIQ is manufactured from "rutin", which exists widely in natural products such as citrus fruits, red beans and buck-wheat, through enzymatic modification. Because EMIQ has high water solubility in addition to antioxidant potential and has been found to be safe in many toxicity studies, such as acute toxicity, 4-week repeated dose toxicity, 13-week repeated dose toxicity, chronic toxicity/carcinogenicity and mutagenicity (Ames test) studies (Tamano et al. 2001), it has been approved as a food additive under the Japan Food Sanitation Law (Japanese Ministry of Health and Welfare 1996). In addition, EMIQ has been self-affirmed Generally Recognized as Safe (GRAS) for use in specific foods in the US, and the US FDA had no questions regarding the conclusion that EMIQ is GRAS as an antioxidant under the conditions of use on the basis of the information provided in the GRAS Notice for EMIQ (US FDA 2007; GRN 000220). EMIQ was also affirmed as GRAS by the Expert Panel of the Flavor and Extract Manufacturers Association (FEMA; Smith et al. 2005; Adams et al. 2007). Recently, Yokohira et al. (2008) reported the possibility that EMIQ is effective as antioxidants in vivo and has a chemopreventive potential against the development of hepatocellular preneoplastic lesion in rats.

Melatonin (MLT; *N*-acetyl, 5-methoxytryptamine) is a chronobiotic, indoleamine neurohormone produced by the pineal gland during darkness in mammals, including humans (Reiter 1991). At both physiological and pharmacological blood concentrations, melatonin plays an important role in a number of physiological and pathophysiological processes including circadian rhythm regulation, sleep disturbances, seasonal reproduction, retinal physiology, immune function and intermediary metabolism (Reiter 1994). In addition, melatonin is known as a powerful scavenger of ROS, such as hydroxyl, peroxyl radicals, singlet oxygen and nitric oxide as well as a stimulator of the antioxidant enzymes: superoxide dismutase, glutathione peroxidase, and catalases, all leading to a decrease in DNA damage and has recently been emphasized as a highly effective molecule in defense against oxidative damage and carcinogenesis (Reiter 1997, 1998, 1999; Blask et al. 1999; Karbownik et al. 2000; Karbownik and Reiter 2000; Reiter et al. 2000).

Anthelmintic benzimidazoles are widely used in meat-producing animals such as cattle, sheep and pigs for the control of endoparasites (Jacobs and Taylor 2001; Velik et al. 2004). Oxfendazole, methyl-5 (6)-phenylsulfinyl-2-benzimidazole carbamate, is a member of the benzimidazole family of anthelmintics. With respect to the hepatocarcinogenicity of OX, it has been reported that OX has no hepatocarcinogenic potential in rats and mice (WHO 1991), but Mitsumori et al. (1997) demonstrated that OX has a liver tumor-promoting activity when the high doses of 250 ppm or more were orally administered to *N*-diethylnitrosamine (DEN)-initiated rats. In addition, Dewa et al. (2009) has recently reported that OX induced sustained production of oxidative stress in the livers of rats, which accounts for, at least in part, the enhancement of microsomal reactive oxygen species (ROS) production and an increased level of oxidative DNA damage as well as lipid peroxidation, and oxidative responses to ROS are generated and its secondary effects might be involved in the liver tumor-promoting mechanism of OX in rats. However, there are few reports dealing with the mechanism of liver tumor-promoting effect of OX (Mitsumori et al. 1997; Dewa et al. 2009).

Many experiments have already shown that MLT has an inhibitory effect on oxidative damage and carcinogenesis, as described earlier. Regarding EMIQ, it has been identified that EMIQ has an antioxidant potential in an in vitro experiment and has a chemopreventive potential against the development of hepatocellular preneoplastic lesions in rats (Yokohira et al. 2008); however, the possible mechanism of the chemopreventive effect of EMIQ is still unknown. Therefore, in the present study, we selected MLT as a positive antioxidant control in the present experiment and selected EMIQ to clarify the possible mechanism of its antioxidant potential on liver tumor promotion induced by OX. We performed experiments using a two-stage hepatocarcinogenesis model in DEN-initiated rats given simultaneously both OX and antioxidants (EMIQ or MLT) and investigated the modifying effect of EMIQ or MLT on liver tumor promotion induced by OX.

Materials and methods

Chemicals and animal and treatments

Oxfendazole, methyl 5-(phenylsulfinyl)-2-benzimidazolecarbamate (OX; 99.7% purity; CAS No. 53716-50-0) and

N-diethylnitrosamine (DEN; CAS No. 55-18-5) were purchased from Hayashi Pure Chemical Industries (Osaka, Japan) and Sigma–Aldrich Chemical Co (St. Louis, MO, USA), respectively. EMIQ (81.5% purity) was kindly supplied by the San-Ei Gen F.F.I. Co., Ltd. (Osaka, Japan). Melatonin [*N*-Acetyl-5-methoxytryptamine, MLT; CAS No. 73-31-4] was purchased from Wako Pure Chemical Industries (Osaka, Japan).

A total of 47 male F344/N Slc rats aged 5 weeks (86.7 ± 6.1 g) were purchased from Japan SLC Inc. (Shizuoka, Japan). The rats were housed in stainless steel cages with 3 or 4 animals per cage and allowed ad libitum access to tap water and a commercial powdered basal diet (MF; Oriental Yeast Industries Co., Ltd., Tokyo, Japan). All the animals were handled under standard conditions (room temperature, 23 ± 3°C; relative humidity, 55 ± 15%; 12-h light and dark cycle).

The experimental design is shown in Fig. 1. We used a two-stage liver carcinogenesis model. After acclimatization, 47 animals were divided into four groups, consisting of 12 (DEN-alone group), 15 (DEN-OX group), 15 (DEN-OX-EMIQ group), 15 (DEN-OX-MLT) and 5 (untreated group) animals. Animals in the DEN-treated groups of DEN-alone, DEN-OX, DEN-OX-EMIQ and DEN-OX-MLT underwent i.p. injection of DEN (200 mg/kg) dissolved in saline to initiate hepatocarcinogenesis. After 2 weeks, the animals in the group of DEN-alone, DEN-OX, DEN-OX-EMIQ or DEN-OX-MLT allowed ad libitum access to tap water, tap water, distilled water containing 2,000 ppm EMIQ or distilled water containing 100 ppm melatonin for 10 weeks, respectively. Additionally, animals in OX-treated groups were fed a diet containing 500 ppm OX for 10 weeks, and animals in DEN-treated groups were subjected to two-thirds of partial hepatectomy 1 week after the treatment of OX and antioxidant substance. Soon after partial hepatectomy, 3, 5, 4 and 3 rats in the groups of DEN-alone, DEN-OX, DEN-OX-EMIQ and DEN-OX-MLT, respectively, died of technical error within 1 week. The dosage of OX in our study was selected based on the results of our previous study (Dewa et al. 2009), but the treatment period was changed to 10 weeks. The dosage of EMIQ was selected based on the previous 13-week toxicity study on EMIQ on the F344/DuCrj rats (Tamano et al. 2001). The dosage of MLT was selected based on the previous report in which a higher dose of MLT (100 ppm in the diet) inhibited the development of hepatocellular adenomas and carcinomas induced by phenobarbital after DEN initiation (Sugie et al. 1998). Diet containing OX were prepared once a week and stored at 4 °C until use. Drinking water containing EMIQ or MLT was prepared once every 2 days. Body weight, food intakes and water intakes were measured once a week. Necropsy was performed under anesthesia with ether at the end of the experiment after starvation for 16 h. The livers of rats were excised and weighed, and the right, right medial and caudate lobes were sliced. One section was fixed in formalin solution for histopathological and immunohistochemical examinations, while the other sections were frozen in liquid nitrogen and stored at −80°C

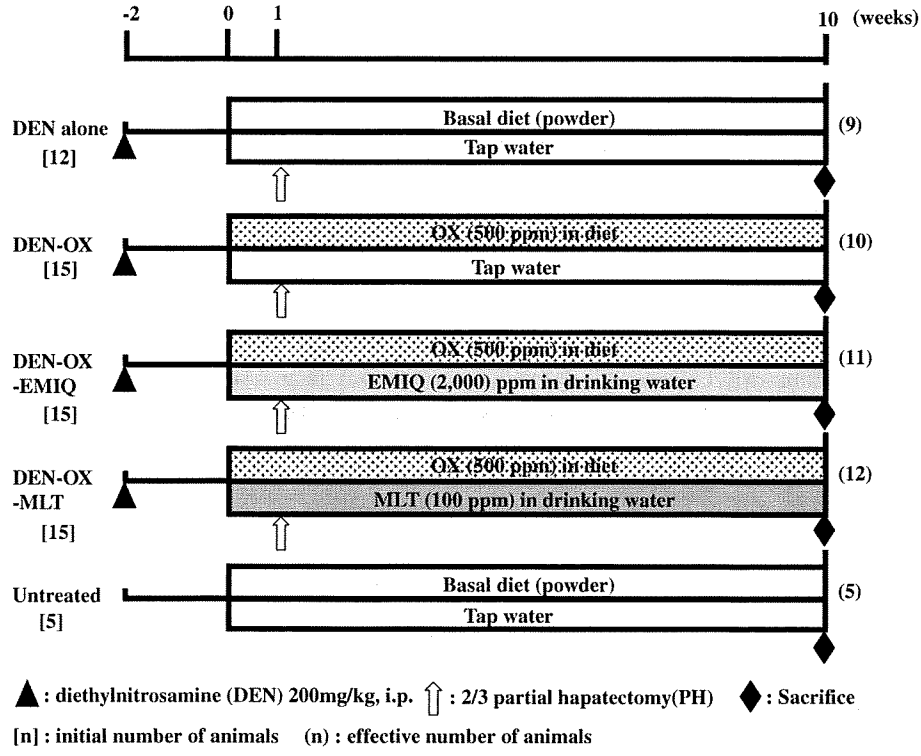

Fig. 1 Experimental design

for future analyses. This study was performed by the method that is appropriate for the animal ethics. Animal care and experiments were carried out in accordance with the Guide for Animal Experimentation of the Tokyo University of Agriculture and Technology conformed to the guidelines for the care and use of laboratory animals as described by the National Institutes of Health. Finally, animals were euthanized by the exsanguinations.

Histological and histochemical examinations

Formalin-fixed liver tissues were embedded in paraffin, and tissue slices were sectioned for histopathological and immunohistochemical examinations. Hematoxylin and eosin (H&E) staining for the sections was conducted according to the routine histopathological methods. In addition, immunohistochemical staining for glutathione *S*-transferase placental form (GST-P) was conducted to evaluate hepatocellular preneoplastic lesions. Tissue sections were stained immunohistochemically using the ABC methods (ABC kit; Nichirei, Tokyo, Japan) for biding of anti-rat GST-P rabbit polyclonal antibody (1:1,000, 4°C, overnight; Medical & Biological Laboratories, Aichi, Japan). All specimens were lightly counterstained with hematoxylin. The numbers and areas of GST-P-positive foci (>0.1-mm diameter) and total areas of the liver sections were measured using WinRoof software (Mitani Corp., Fukui, Japan).

RNA isolation and gene expression analyses

Total RNA was isolated from 5 animals of each group using the TRIzol reagent (Invitrogen, Corp., CA, USA) and the SuperScriptIII First-Strand Synthesis System (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's protocol. Quantitative real-time RT–PCR analyses with SYBR green PCR master mix (Applied Biosystems, Foster City, CA, USA) using an ABI Prism 7000 Sequence Detection System (Applied Biosystems) was performed to compare mRNA expression levels of oxidative stress-related genes reported by Dewa et al. (2009). The relative differences in gene expression were calculated using cycle time (Ct) values that were first normalized to those of the hypoxanthine–guanine phosphoribosyltransferase (*Hprt*) gene, the endogenous control in the same sample, and then relative to a control Ct value by the 2-ddCt method (Livak and Schmittgen 2001). The PCR primers were used references from the previous study (Dewa et al. 2009). These genes were cytochrome P450, family 1, subfamily a, polypeptide 1 (*Cyp1a1*); *Cyp1a2*; *Cyp2b2*; NAD(P)H dehydrogenase, quinone 1 (*Nqo1*); glutathione *S*-transferase, mu 1 (*Gstm1*); glutathione peroxidase 2 (*Gpx2*); aldo–keto reductase family 7, member A3 (*Akr7a3*); glutathione *S*-transferase Yc2 subunit (*Yc2*); malic enzyme 1 (*Me-1*).

Determination of microsomal ROS production of the liver

Dewa et al. (2009) reported that OX exhibits tumor-promoting activity that enhances preneoplastic foci in a DEN-initiated hepatocarcinogenesis model in partially hepatectomized rats, showing that oxidative stress such as ROS production generated from microsomes isolated from the livers of OX-treated rats was one of the main factors as the mechanism of OX-induced hepatocarcinogenesis. Therefore, in the present study, we evaluated the effect on ROS production using the non-fluorescent probe, 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA; Molecular Probes), which is known as a sensitive probe for detecting ROS (Serron et al. 2000), in the case that EMIQ or melatonin was added in the microsomes isolated from the livers of rats of DEN-OX group. The rat liver tissues obtained from DEN-OX group were homogenized in ice-cold 1.15% KCl buffer (pH 7.4) containing 0.2 mM EDTA, 0.1 mM DTT, 0.1 mM PMSF and 20% glycerin. Hepatic microsomes obtained from the homogenates were routinely purified by differential centrifugation (Sequeira et al. 1992), suspended in the earlier mentioned buffer. ROS was measured by partially modifying the method of Serron et al. (2000). Microsomes (Final conc. 0.15 mg/mL) were incubated in the dark at 37°C in 40 mM Tris buffer (pH 7.4) and 5 μM DCFH-DA. At the end of the incubation period, EMIQ (Final conc. 0.001–1 mM), MLT (Final conc. 0.003–30 μM), H_2O_2 (Final conc. 1 mM; positive control), SKF-525-A (Final conc. 0.1 mM; positive control, Toronto Research Chemicals, ON, Canada) or the vehicle were added and additionally were incubated at 37°C for 30 min under dark. Following that, nicotinamide adenine dinucleotide phosphate (NADPH; Final conc. 0.6 mM) was added, and the mixture was incubated at 37°C for 30 min under dark. The rate at which ROS formed the fluorescent product was measured using a microplate reader (excitation 485 nm; emission 528 nm). The data was normalized to control values, and the control was expressed as a value of 100%.

Statistical evaluation

Statistical analyses were performed using SAS statistical software (SAS Institute, Inc., Cary, NC), and all results are presented as mean ± SD. Multigroups were used to test the homogeneity of variance between the groups by using Bartlett's test. When the data were homogenous, Dunnett's test was used, and when heterogeneous, Dunnett's rank sum test was used. Dunnett's multiple comparison tests were performed between DEN-alone group and OX-treated groups, and between DEN-OX group and antioxidant-treated groups. With respect to the analyses of microsomal ROS production, the statistical significance of differences between vehicle control and antioxidant-treated samples