

Quercetin acts as an antioxidant and downregulates CYP1A1 and CYP1B1 against DMBA-induced oxidative stress in mice

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Abstract. We investigated the effects of quercetin on 7,12-dimethylbenz(a)anthracene (DMBA)-induced oxidative stress and the expression of CYP1A1 and CYP1B1 in mice. Quercetin was administered orally to mice at 100 or 250 mg/kg BW for 18 days, after which DMBA (34 mg/kg BW) was administered intragastrically twice. Quercetin showed side effects such as increased aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in DMBA-untreated mice. Also, quercetin induced AST and ALT in DMBA-treated, although this was not significantly different from levels in DMBA-treated controls. The thiobarbituric acid reactive substances (TBARS) value showed a tendency to decrease following quercetin treatment; these decreases were significantly greater in the DMBA-treated compared to the untreated groups. Also, catalase and superoxide dismutase (SOD) activities as well as their mRNA expression were increased by quercetin; this increase was more pronounced in DMBA-treated compared to untreated mice. DMBA induced CYP1 activity as well as expression of CYP1A1 and CYP1B1. Each of these effects was significantly reduced by quercetin; however, this reduction was observed for CYP1A1 at only the higher dose and for CYP1B1 at both doses. These data suggest that quercetin shows antioxidant activity against DMBA-induced oxidative stress. Moreover, its regulation of CYP1A1 and CYP1B1 suggests the potential of quercetin as an anticancer supplement.

Introduction

Quercetin (3,3',4',5,7-pentahydroxyflavone), a member of the flavone family, is found in many foods, including vegetables, tea, fruit and wine (1-3). Several epidemiological studies have supported the hypothesis that the antioxidant action of quer-

etin may reduce the risk of developing cancer (4,5). Based on its anti- and prooxidant activities, two anticancer mechanisms of quercetin have been proposed. First, quercetin can scavenge reactive oxygen species (ROS) as an antioxidant and thereby reduce DNA damage (6,7). In addition, quercetin has been shown to affect the cytochrome P450 (CYP) system, which is one of the main sources of ROS generation in the cell (8,9). On the other hand, quercetin can generate ROS by autoxidation and redox-cycling and so act as a prooxidant (10,11). Several researchers have suggested that the biphasic functions of quercetin may be explained by a concentration-dependent effect; that is, antioxidant activity occurs at lower concentrations than the prooxidant activity (12-14). Our previous report also suggests that quercetin may act not only as an antioxidant, but also as a prooxidant (15,16). The prooxidant activity of quercetin is now becoming accepted as contributing to cancer chemotherapy and inhibition of tumor growth, and increasing evidence suggests that quercetin shows specific inhibition of cancer cell growth (17-19). Consistent with that finding, our report indicated that quercetin significantly inhibited cellular proliferation and induced cell cycle arrest and apoptosis in human breast cancer *in vivo* (20) and *in vitro* (21).

In the present study, we focused on the anti- or prooxidant effect of chronic quercetin administration at high doses under oxidative stress condition, i.e., mice treated with 7,12-dimethylbenz(a)anthracene (DMBA). As a potent procarcinogen, DMBA requires metabolic conversion to its ultimate carcinogenic metabolites by oxidation, which is conducted by CYP1A1, CYP1A2 and CYP1B1 (22). In addition, DMBA induces substantial oxidative damage as a result of the formation of ROS such as peroxides, hydroxyl, and superoxide anion radicals in organs such as the liver and mammary glands (23,24).

Materials and methods

Quercetin treatment and sample preparation. Female ICR mice (23-25 g; Central Laboratory Animal Inc., Seoul, Korea) were housed in groups of three in polypropylene cages (22±2°C, 40-50% relative humidity) under controlled lighting (12-h light/dark cycle). Mice were fed an AIN-93M diet (Dyets, Bethlehem, PA, USA) and allowed free access to water. After an acclimation period, mice were divided into two groups, one of which was maintained on a vehicle and another that was intragastrically administered a dose of 34 mg

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Table I. Food intake and body weight.

Group classification	Food intake (g/day)	Initial body weight (g)	Final body weight (g)
DMBA-untreated mice			
0 mg/kg quercetin	3.54±0.52	24.3±1.1	30.5±2.31
100 mg/kg quercetin	4.01±0.36	25.3±1.0	31.2±3.36
250 mg/kg quercetin	3.46±0.41	23.6±1.1	29.5±3.42
DMBA-treated mice			
0 mg/kg quercetin	3.46±0.58	24.3±1.0	28.2±3.36
100 mg/kg quercetin	3.53±0.63	24.5±1.0	31.5±3.28
250 mg/kg quercetin	3.39±0.41 ^a	25.4±0.9	27.6±4.32

Mice were divided into two groups, one was maintained on a vehicle and the other that was intragastrically administered DMBA. Subsequently, each group was subdivided randomly into three treatment groups, two of which received quercetin at 100 and 250 mg/kg body weight for 18 days. Values are the mean ± SD (n=5-7). ^aP<0.05, significantly different from the respective control group (DMBA-treated or untreated).

DMBA/kg body weight (BW) in corn oil vehicle twice a week. Subsequently, each group was subdivided randomly into three treatment groups. Quercetin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in corn oil and administered orally to two of the three groups at 100 and 250 mg/kg body weight for 18 days, respectively. Mice were treated with quercetin or the vehicle and weighed at the same time (7 to 8 am) every morning for 18 days. Quercetin was stored in a refrigerator. Quercetin suspension was freshly prepared once every three days based on the body weight of the animals and stored at -4°C to 0°C until used. Animal care in this study conformed to the Guide for the Care and Use of Laboratory Animals published by the USA National Institutes of Health.

At the end of the experiment, mice were rapidly anesthetized using ether at 6 h after final administration of quercetin. After blood was taken from the heart by heart puncture, serum was obtained by centrifuging the blood at 2,500 rpm for 15 min. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) contents were determined by the Advia 1650 chemistry analyzer (Siemens Medical Solutions Diagnostics, Norwood, MA, USA) using appropriate kits (Bayer). In addition, their livers were isolated, blotted, weighed, frozen in liquid nitrogen and stored at -70°C until assayed. Each sample homogenate was centrifuged at 800 x g to remove cell debris and nuclei; the supernatants were centrifuged at 10,000 x g for 10 min and the portions of the post-mitochondrial fraction were transferred. To obtain the cytosolic fractions for superoxide dismutase (SOD) activity measurement, the remaining supernatant was centrifuged further at 105,000 x g for 45 min using a 50 Ti Rotor in a Beckman model L90 ultracentrifuge. Post-mitochondrial and cytosolic fractions were stored at -80°C in aliquots until analysis within one week.

Determination of oxidative stress biomarkers and activity of antioxidant enzymes. The TBARS (thiobarbituric acid reactive substances) value was determined by measuring the concentration of malondialdehyde according to the method of Ohkawa *et al* (25) and calculated according to the molar absorption coefficient of malondialdehyde (MDA), $\epsilon = 1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ at 535 nm. Catalase activity was assayed by the method of

Aebi (26) and was expressed as nmol/min/mg/protein of decomposed H₂O₂. SOD activity was assayed according to the pyrogallol autoxidation method of Marklund and Marklund (27). Each unit of SOD activity was defined as the quantity of enzyme that inhibited autoxidation of pyrogallol by 50% under experimental conditions.

Assay of CYP enzyme activity. CYP activity was determined by formation of resorufin (Sigma) from ethoxyresorufin. Microsomal proteins (100-250 µg) were mixed with 100 mM phosphate buffer (pH 7.4) and 1-2 µM of 7-ethoxyresorufin. The reaction was initiated by the addition of 0.50 mM NADPH and was incubated at 37°C for 10 min and scanned in the microtiter plate fluorescent reader. The reaction was stopped by the addition of methanol, and then after centrifugation. The amount of resorufin formed was quantified from a standard curve constructed with known amounts of resorufin. The excitation and emission wavelengths for the detection of resorufin were 530 and 590 nm, respectively.

Immunoblotting assay. Proteins (30 µg/well) denatured with sample buffer were separated by 10% SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto nitrocellulose membranes (0.45 µm). The membranes were blocked with a 1% BSA solution for 3 h and washed twice with PBS containing 0.2% Tween-20, and incubated with the primary antibody overnight at 4°C. Antibodies against catalase, CuZn-, Mn-SOD, CYP1A1, CYP1B1, aryl hydrocarbon receptor (AhR) and β-actin were purchased from Santa Cruz Biotechnology, Inc., (Santa Cruz, CA, USA) and used to probe the separate membranes. The next day, the immunoreaction was continued with a secondary goat anti-rabbit horseradish-peroxidase-conjugated antibody after washing for 2 h at room temperature. The specific protein bands were detected by Opti-4CN Substrate kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analyses. All data were expressed as percent compared with vehicle-treated control, which were arbitrarily assigned 100%. All values are expressed as mean ± SD. Data

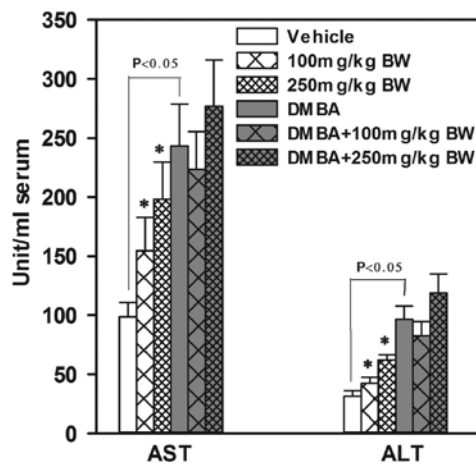


Figure 1. Effect of quercetin administration on serum AST and ALT levels. Values are mean \pm SD (n=5-7). *P<0.05, significantly different from the respective control group (DMBA-treated or untreated).

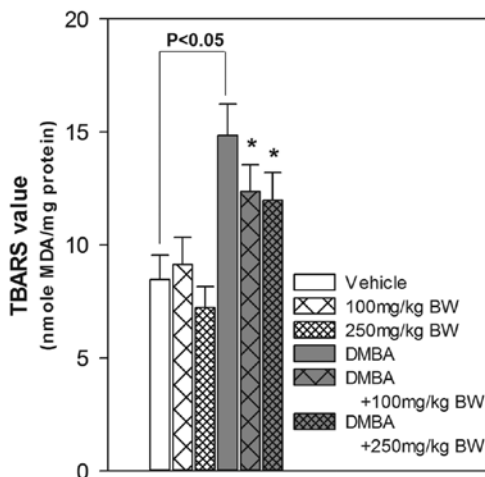


Figure 2. Effect of quercetin administration on hepatic TBARS values. Values are mean \pm SD (n=5-7). *P<0.05, significantly different from the respective control group (DMBA-treated or untreated).

were analyzed by the unpaired Student's t-test or one-way analysis of variance followed by the Dunnett's multiple comparison test (SigmaStat, Jandel, San Rafael, USA). For all comparisons, differences were considered statistically significant at P<0.05.

Results

Food intake and body weight. Although no statistically significant differences were recorded between DMBA-untreated and DMBA-treated mice in food intake and body weight gain, DMBA induced a 9.2% decrease in final body weight compared to DMBA-untreated controls (Table I). Also, quercetin at the higher dose caused a non-significant decrease in the final body weight in both DMBA-untreated and -treated mice.

Serum AST and ALT levels and hepatic TBARS value. Serum AST and ALT levels were significantly increased by 2.5- and 3.1-fold DMBA-treated and -untreated control mice, respec-

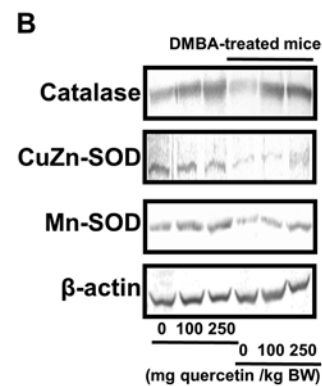
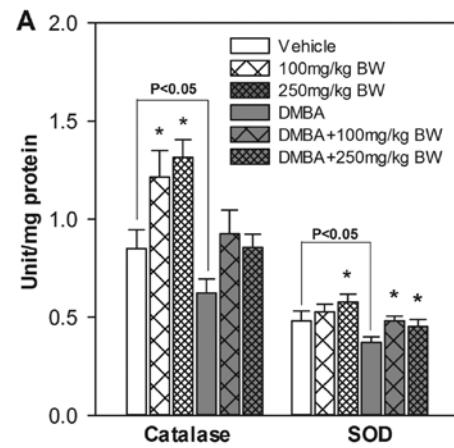


Figure 3. Activities (A) of catalase and total SOD, and its protein expression (B). Values are mean \pm SD (n=5-7). *P<0.05, significantly different from the respective control group (DMBA-treated or untreated).

tively (Fig. 1). Although quercetin administration significantly increased AST and ALT levels in a dose-dependent manner in DMBA-untreated mice, quercetin at the lower (100 mg/kg BW), but not the higher dose (250 mg/kg BW) inhibited both AST and ALT (8.2 and 14.2% inhibition) in DMBA-treated mice, which was increased by DMBA. DMBA-treated mice given quercetin at the higher dose showed 13.6 and 23.0% increase in ALT and AST levels, respectively, although this difference was not significant.

The hepatic TBARS value of DMBA-treated mice was significantly (P<0.05) greater than that of untreated mice (8.47 and 14.85 nmol/mg protein, respectively, Fig. 2). Quercetin at both doses significantly decreased the TBARS value in DMBA-treated mice, but not untreated mice (16.7 and 19.4% decreases at 100 and 250 mg/kg BW, respectively, P<0.05). Although quercetin at the higher dose (250 mg/kg BW) decreased the TBARS value in DMBA-untreated mice (14.6% compared to the control group), this difference was not significant.

Activity and expression of the antioxidant defense enzymes, catalase and total SOD. The hepatic catalase activity in DMBA-treated mice was significantly (P<0.05) lower than in untreated mice (0.62 and 0.85 U/mg protein, respectively, Fig. 3A). Quercetin increased catalase activity in all mice (both of DMBA-untreated and -treated) regardless of quercetin. Total SOD activities in DMBA-treated mice were also significantly (P<0.05) decreased by about 23% compared to those of untreated control mice. Although quercetin had no

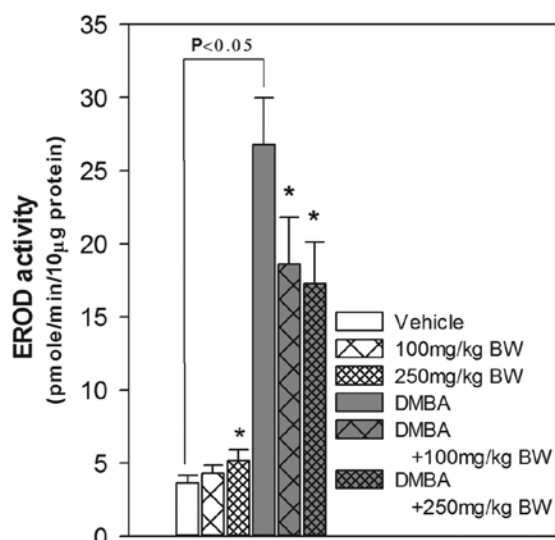


Figure 4. Effect of quercetin administration on ethoxyresorufin O-deethylase (EROD) activity. Values are mean \pm SD (n=5-7). *P<0.05, significantly different from the respective control group (DMBA-treated or untreated).

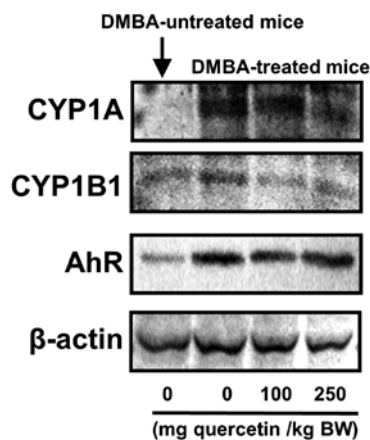


Figure 5. Effect of quercetin administration on expression of CYP1A1, CYP1B1 and AhR in DMBA-treated mice.

significant affect under normal conditions, the decreased total SOD in DMBA-treated mice returned to normal levels after quercetin administration.

A similar pattern was evident for the protein expression (Fig. 3B). Increases in Mn-SOD expression by quercetin were more pronounced than those of CuZn-SOD in DMBA-treated mice.

Ethoxyresorufin O-deethylase (EROD) activity and CYP1A1, CYP1B1 and AhR expression. The basal activity of EROD in DMBA-treated mice was significantly greater than that in untreated controls (26.78 and 3.65 pmol/min/10 µg protein, Fig. 4). Quercetin non-significantly increased EROD activity in normal mice, while the increased EROD level caused by DMBA was significantly decreased by quercetin (by 30.5 and 35.5% with 100 and 250 mg/kg quercetin, respectively).

To verify EROD activity, the effect of quercetin on CYP1A1, CYP1B1 and AhR expression was quantified in DMBA-treated mice (Fig. 5). In control mice, CYP1A1, CYP1B1, and AhR

expressions were dramatically increased compared to those of DMBA-untreated mice. Activated CYP1A1 was decreased after quercetin administration at the higher dose and CYP1B1 was decreased in both doses. AhR expression was not affected by quercetin administration.

Discussion

Quercetin is one of the most abundant and most powerful antioxidant of over 6,000 flavonoids. Recently, the role of quercetin has been suggested to depend on its concentration and exposure period (12,15). Moreover, its safety remains controversial because several studies have warned that the use of quercetin at high concentrations has deleterious effects.

In the present study, the dose of quercetin used (100 and 250 mg/kg BW/day) was much higher than that found in human foods, which typically contain 1-10 mg/g edible plant matter (28-30). Administration of quercetin resulted in significant increases in serum AST and ALT levels. These may have resulted from leakage of these enzymes into the circulation, and may indicate liver damage and altered liver function (31,32). However, quercetin (100 mg/kg BW) in mice treated with DMBA decreased serum AST and ALT levels, although this difference was not significant.

Oxidative stress produces deleterious effects by initiating lipid peroxidation either directly or by acting as second messengers for the primary free radicals that initiate lipid peroxidation (33). The TBARS value (a biomarker of lipid peroxidation) was significantly higher in the DMBA-treated group than in the untreated control group. This is consistent with previous reports stating that DMBA induces critical oxidative damage in the liver *in vivo* (34-36). Even though quercetin did not reduce the TBARS value of DMBA-untreated mice to normal levels, quercetin has previously been shown to inhibit lipid peroxidation.

SOD is the first and most important line of antioxidant enzyme defense against oxidative stress, particularly oxygen radicals. SOD scavenges superoxide by converting it to peroxide, which is then destroyed by catalase (37). Catalase and total SOD activity were significantly decreased in DMBA-treated mice compared to untreated controls, suggesting the induction of oxidative stress by DMBA. The reduced catalase and total SOD activities were increased by quercetin administration to normal levels (DMBA-untreated control). This trend was also observed in the expression of catalase and SOD (Mn- and CuZn-SOD).

Based on these results, although quercetin acts as an antioxidant by either decreasing TBARS or increasing catalase and SOD activities, chronic high consumption results in obvious side effects. One possible explanation is that quercetin at higher doses may be more active under conditions of oxidative stress. A natural dynamic balance exists between the amount of free radicals generated and the antioxidant defense system, which protects the body against carcinogenesis (38). Many antioxidants have been studied for possible use as anticancer agents. Evidence exists that flavonoids can enhance the detoxification of carcinogens and decrease DNA damage (39,40). Thus, notwithstanding some side effects, quercetin at higher doses under DMBA-induced oxidative stress conditions may have potential as an antioxidant agent.

DMBA is a polycyclic aromatic hydrocarbon (PAHs) and is oxidized by CYP1A1 and CYP1B1 in the liver to form carcinogenic metabolites, including diol epoxides and other toxic reactive oxygen radicals (41,42). *In vitro* experiments indicate that DMBA in rat liver microsomes causes dose- and time-dependent production of H₂O₂, an oxidant closely associated with tumor promotion (43,44). The induction of such CYP1 family members as CYP1A1, 1A2, and 1B1 is regulated by the aryl hydrocarbon receptor (AhR) and the AhR nuclear translocator (ARNT).

Consistent with these facts, the expression of CYP1A1 and CYP1B1 in DMBA-treated mice was dramatically increased. A similar pattern was observed in EROD activity, which was used to determine the level of CYP activity. DMBA strongly induced EROD activity in DMBA-treated mice as well as expression of CYP1A1, CYP1B1 and AhR. The induction of CYP1 family members such as CYP1A1, 1A2 and 1B1 is regulated by AhR (45,46). Quercetin was reported to significantly downregulate the CYP system both *in vitro* (47-49) and *in vivo* (50,51). The increase in CYP1A1 and CYP1A2 levels induced by oxidative products was inhibited by quercetin (47-49). In the present study, as a result of quercetin administration at the higher dose (200 mg), CYP1A1 and CYP1B1, but not AhR, levels, were significantly decreased. These results suggest that downregulation of CYP1A1 by quercetin is irrelevant to the regulation of AhR in mice treated with DMBA. Thus, quercetin has the potential to be applied as a chemotherapeutic agent, since the discovery of molecules able to modulate the activity of specific CYP isomers is a major goal in the development of chemotherapeutics.

In summary, the study demonstrated that chronic administration of high-dose quercetin may have a dual effect. The regular consumption of quercetin-enriched supplements should be monitored carefully because of their potential side effects. Nevertheless, the activation of antioxidant-related enzymes and regulation of CYPs by quercetin under oxidative stress conditions shows the possibility of quercetin as an anticancer chemotherapeutic without causing side effects.

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