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RESEARCH NOTE

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Equol Modulates Induction of Hepatic CYP 1A1, 1B1, and AhR in Mice Treated with 7,12-Dimethylbenz(a)anthracene

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Abstract Present study was investigated the hepatic effects of equol on the 7,12-dimethylbenz(a)anthracene (DMBA)-induced enzymatic activity and expression of CYP1A1 and CYP1B1 in mice. Equol was administered orally at 5 and 25 mg/kg BW for 4 weeks. Subsequently, mice pretreated with equol received DMBA intragastrically twice a week for 2 weeks. DMBA induced CYP1 activity as well as the expression of CYP1A1 and CYP1B1. Each of these effects was significantly reduced by equol in dose-dependent manner ($p<0.05$). Equol also reduced the relative AhR mRNA expression, similar to its effect on CYP1A1. These results suggest that equol modulates the CYP1A1 through a reduction of AhR expression in mice treated with DMBA.

Keywords: aryl hydrocarbon receptor (AhR), cytochrome P450 enzyme, 7,12-dimethylbenz(a)anthracene (DMBA), equol, *in vivo*

Introduction

Equol [7-hydroxy-3-(4'-hydroxyphenyl)-chroman] is a bioactive metabolite of daidzein that is formed by intestinal bacteria. It is thought to have an effect on human health similar to that of the isoflavones genistein and daidzein. Several reports have suggested that equol has the greatest bioactivity of all isoflavones tested when measured *in vitro* (1,2). It has been suggested that it may have great potential as a cancer chemopreventive agent as well as isoflavones. The anticancer effects of isoflavones were attributed to estrogenic/antiestrogenic activity, the induction of cell-cycle arrest and apoptosis, the inhibition of oxidative stress, and the activation of cell death signaling (3-6). In addition, isoflavones have been shown to affect the cytochrome P450 (CYP) system, which is an ongoing target of cancer drug development (7,8). The discovery of molecules able to modulate the activity of specific CYP isomers is a major goal in the development of chemotherapeutics (9-11). For example, some flavonoids modulate CYP-encoding genes either by direct ligand interaction with the aryl hydrocarbon receptor (AhR) or by specialized xenobiotic activated receptors (XARs), which include the AhR (12).

Until recently, the biological effects of equol, the major metabolite of daidzein, are not as well understood as those of daidzein itself. Therefore, to investigate the anticancer effect of equol via modulation of CYP system, this study was designed to analyse the expression of hepatic CYP1A1 and CYP1B1 in mice treated with chemical carcinogen 7,12-dimethylbenz(a)anthracene (DMBA) following 4 weeks of treatment with equol. As a potent procarcinogen, DMBA requires metabolic conversion to its ultimate carcinogenic metabolites by oxidation, which is conducted by CYP1A1 and 1B1. In addition, DMBA induces substantial oxidative damage as result in the formation of reactive oxygen species (ROS) such as peroxides, hydroxyl, and superoxide anion radicals in organs such as the liver and mammary glands (13,14).

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Materials and Methods

Equol treatment and sample preparation Female ICR mouse (23-25 g; Central Lab. Animal Inc., Seoul, Korea) were housed 6 to a polypropylene cage (22±2°C, 40-50% relative humidity) under controlled lighting (12-hr light/dark cycle). Mice were fed an AIN 93M diet (Dyets, Bethlehem, PA, USA) and allowed free access to water. After an adaptation period, mice were divided randomly into 4 treatment groups. Equol (LC Laboratories®, Woburn, MA, USA) was dissolved in water and administered orally to 2 groups at 5 and 25 mg/kg body weight (BW) for 4 weeks. Subsequently, the DMBA-treated groups were intragastrically administered a dose of 34 mg/kg BW in corn oil vehicle twice a week for 2 weeks. The dose and timing of DMBA treatment were selected based on the values shown in previous reports (15,16) to induce CYP expression most efficiently. Animal care in this study conformed to the 'guide for the care and use of laboratory animals' published by the U.S. National Institutes of Health. Twenty-four hr after the last DMBA treatment, mice were anesthetized with ether. Microsomes of liver were prepared by differential centrifugation within 24 hr. Each liver were homogenized for about 60 sec in 1 mM tetraacetic acid disodium salt (EDTA), 250 mM sucrose, and 50 mM Tris-acetate buffer, pH 7.4. Homogenates were centrifuged at 4°C for 30 min at 10,000×g. The microsomes were obtained by centrifugation of the supernatant at 101,000×g for 60 min at 4°C using a 50 Ti rotor in a Beckman model L90 ultracentrifuge.

Ethoxyresorufin-*O*-deethylase (EROD) assay CYP activity was determined by formation of resorufin (Sigma-Aldrich, St. Louis, MO, USA) from ethoxyresorufin.

Microsomal proteins (approximately 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 centrifuged. 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 Microsomal proteins (10 μg/well) denatured with sample buffer were separated by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel. Proteins were transferred onto nitrocellulose membranes (0.45-μm). The membranes were blocked with a 1% bovaine serum albumin (BSA) solution for 3 hr and washed twice with Phosphate buffered saline (PBS) containing 0.2% Tween-20, and incubated with the primary antibody overnight at 4°C. Antibodies against CYP 1A1, 1B1, and β-actin were purchased from Santa Cruz (Santa Cruz Biotechnology, Inc., CA, USA) and used to probe the separate membranes. The next day, the immunoreaction was continued with the secondary goat anti-rabbit horseradish-peroxidase-conjugated antibody after washing for 2 hr at room temperature. The specific protein bands were detected by Opti-4CN Substrate kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Relative mRNA expression by real time-polymerase chain reaction (RT-PCR) Samples were homogenized with Trizol (Gibco BRL, Gaithersberg, MD, USA) and mRNA was extracted according to the manufacturer's protocol. First-strand cDNA was synthesized using SuperScript First-Strand Synthesis system (Gibco BRL). Each target mRNA expression was quantified by RT-PCR with the use of CFB-3120 MIniOpticon™ system (Bio-Rad). CFB-3120 MIniOpticon™ system uses an array of 48-light emmitting diodes (LED)s, which is efficiently excite fluorescent dyes with absorption spectra in the 470-505 nm range. PCR reactions were carried out with 2X SYBR® Green mix (Finnzymes, Espoo, Finland). Each mRNA levels were calculated by means of the comparative cycle threshold (C_t) method using $2^{\Delta\Delta Ct}$ according to the manufacturer's instructions. Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control (internal control). The fold change in target gene relative to the endogenous control was determined as Fold change=$2^{-\Delta\Delta Ct}$;where $\Delta\Delta Ct=(Ct_{target}-Ct_{endogenous})_{treated\ group}-(Ct_{target}-Ct_{endogenous})_{control\ group}$. The untreated sample (control group) was defined as the calibrator in this experiment. Therefore, the amounts of AhR transcripts in the other samples were assigned dimensionless numbers relative to the levels in the calibrator sample.

Statistical analyses Data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple-comparison test (Sigma Stat, Jandel, San Rafael, CA, USA). For all comparisons, differences were considered statistically significant at $p<0.05$.

Results and Discussion

Recently, equol has been considered to be an important player in the bioactive mechanism of isoflavones such as daidzein, although little information has yet been reported. It has been reported that daidzein may be an effective agent against cell growth in some cancer cell types (17,18). This suggesting is consistent with that equol apparently has also antiproliferative effects (19-21) similar to its pro-drug daidzein.

Effect of equol on CYP1 activity as well as the expression of CYP1A1 and CYP1B1 in mice treated with DMBA CYPs enzymes catalyze the oxidation of endogenous and exogenous substrates. They also play major roles in determining the half-life of many therapeutic agents. The physiological effects of these enzymes include catabolizing drugs to their inactive metabolites and converting prodrugs to their active forms (8,22). DMBA is a polycyclic aromatic hydrocarbon (PAHs) that has been shown to induce mammary carcinogenesis in an animal model. It has also been used to study the initiation, progression, pathogenesis, and prevention of human breast carcinoma (23). DMBA is oxidized by CYP1A1 and CYP1B1 in the liver to form carcinogenic metabolites, including diol epoxides and other toxic reactive oxygen radicals (24-26). Consistent with these facts, we observed a 5.27- and 4.28-folds increase in the expression of CYP1A1 and CYP1B1, respectively, in livers of DMBA-treated mice compared to control mice (Fig. 1A). Equol administration significantly reduced the increase in CYP1A1 by DMBA in a dose-dependent manner. At 5 and 25 mg/kg BW, equol reduced the expression of CYP1A1 by 26.19 and 68.88%, respectively, compared with the expression observed in the DMBA group. Moreover, equol decreased the expression of CYP1B1 by 14.75 and 29.62% at 5 and 25 mg/kg BW, respectively, compared to the level in the DMBA group, although changed level of CYP 1B1 expression was smaller than that observed for CYP1A1 expression. Similar pattern was observed in the results of an EROD activity assay, which was used to determine the level of CYP activity (Fig. 1B). DMBA strongly induced EROD activity in livers of DMBA-treated mice as well as the expression of CYP1A1 and CYP1B1. Equol administration significantly inhibited the CYPs activity (27.99 and 43.92% at 5 and 25 mg/kg BW compared to the control group, respectively, $p<0.05$).

Effect of equol on relative AhR mRNA expression in mice treated with DMBA The induction of such CYP1 family members as CYP1A1, 1A2, and 1B1 is regulated by AhR and aryl hydrocarbon receptor nuclear translocator (ARNT). Upon binding DMBA, AhR translocates to the nucleus where it dimerizes with ARNT. The AhR-ARNT heterodimer functions as a ligand-activated transcription factor, binding xenobiotic response elements (XREs) in the CYP1 promoter (27,28). In the present study, the AhR relative mRNA expression was significantly increased by approximately 2.33-fold in DMBA control mice compared to control mice (Fig. 2). As a result of equol administration,

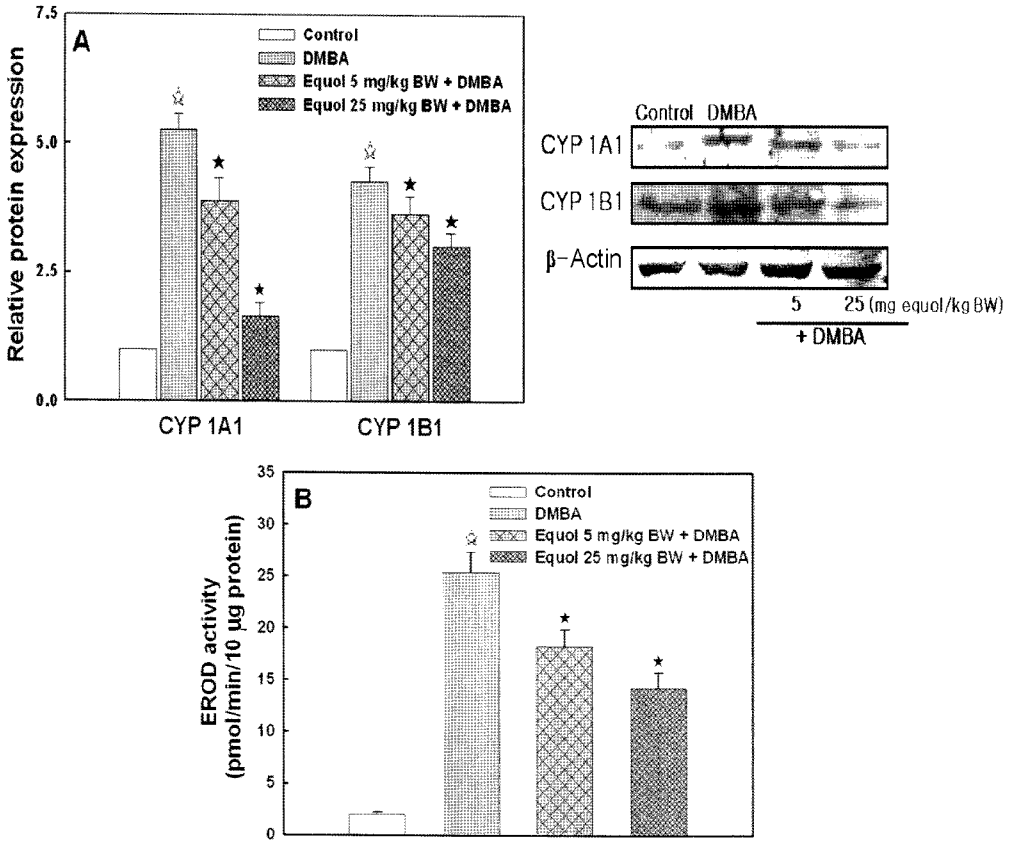

Fig. 1. Effect of equol on expression (A) of CYP 1A1 and CYP 1B1 and activity (B) of CYPs. Values are expressed mean±SD (n=6). For relative expression density, the protein expressions were calculated relative to β-actin and the value for the respective control group was accepted to be '1.0'. $^{\star}p<0.05$, significantly difference between control and DMBA group. $^{\star}p<0.05$, significantly different from the DMBA group.

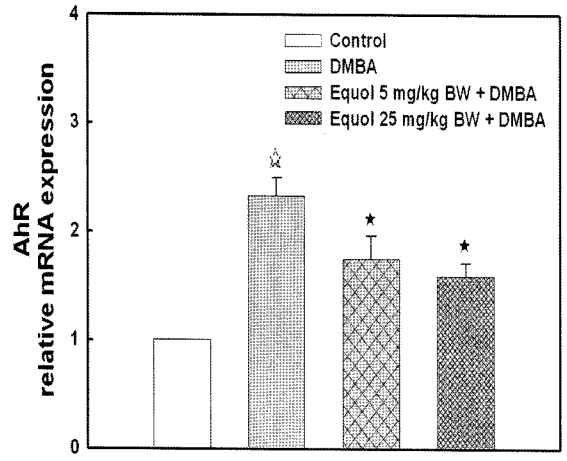

Fig. 2. Effect of equol on aryl hydrocarbon receptor (AhR) mRNA expression. Values are expressed mean±SD (n=6). Level of control group was accepted to be '1.0'. $^{\star}p<0.05$, significantly difference between control and DMBA group. $^{\star}p<0.05$, significantly different from the DMBA group.

the mRNA expression of AhR was significantly decreased (24.89 and 31.76% at 5 and 2 mg/kg BW, respectively) compared with the level of expression observed in the DMBA group. These results suggest that a reduction in AhR expression by equol is associated with down-regulated CYP1A1 in mice treated with DMBA. These modulations may explain the anticancer effects of equol, although the precise mechanism underlying this action of equol is unclear.

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