Anticancer mechanism of equol in 7,12-dimethylbenz(a)anthracene-treated animals

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Abstract. This study investigated the anticancer effects of equol, the major metabolite of the antioxidant phytochemical daidzein, on 7,12-dimethylbenz(a)anthracene (DMBA)-treated animals and explored its anticancer mechanism. The experiment consisted of two parts. In the first part, Sprague-Dawley rats were given equol daily at 5 and 25 mg/kg body weight (BW) for 8 weeks after a single dose of DMBA (100 mg/kg BW). As a control, rats were divided into vehicle alone and DMBA alone groups. Equol administration at a higher dose effectively suppressed tumor formation and PCNA overexpression. The activation of p53 by equol subsequently affected the cyclin-dependent kinase inhibitor p21Cip1. This was associated with equol-induced apoptosis in mammary gland tumors, as evidenced by the decreased Bcl-2 expression and increased Bax expression, together with the activation of caspase-3 and poly(ADP-ribose) polymerase (PARP). In the second part, oral pre-administration of equol to mice which received DMBA intragastrically twice a week for 2 weeks significantly decreased their levels of biomarkers (thiobarbituric acid-reactive substances, carbonyl content and serum 8-hydroxy-2-deoxyguanosine) of DMBA-induced oxidative stress. Although several antioxidant enzymes were downregulated in mice treated with DMBA alone, pre-administration of equol blocked much of this effect, increasing catalase and superoxide dismutase activity in a dose-dependent manner. Although equol did not affect the ratio of oxidized to reduced glutathione, it activated the glutathione peroxidase and glutathione reductase enzymes, and this effect was significant at a dose of 25 mg equol/kg body weight. DMBA treatment induced apoptosis, as shown by a decrease in the Bcl-2 levels and an increase in the levels of Bax, cleaved caspase-3 and poly(ADP-ribose) polymerase. These apoptotic effects were also reversed by equol at all doses tested. Based on these results, equol possesses anticancer activity that suppresses tumor formation via apoptosis induction in rats with DMBA-induced mammary gland tumors. In addition, equol showed a hepatic protective effect by acting as an antioxidant and by reducing apoptosis.

Introduction

Equol [7-hydroxy-3-(4'-hydroxyphenyl)-chroman] is a bioactive metabolite of isoflavone daidzein that is formed by intestinal bacteria. More recently, several researchers have suggested that equol is considered to be an important player in the bioactive mechanism of isoflavones such as daidzein (1,2), although little information has yet been reported.

Isoflavones such as genistein and daidzein, which are found in fruits, nuts, soybeans and soy-based products (3), have been extensively studied for their antioxidant properties. In biological systems, antioxidants scavenge and block reaction oxygen species (ROS), which are thought to promote the development of chronic, inflammatory, degenerative and age-related diseases (4-6). To protect tissues from oxidative damage caused by ROS, the human body has antioxidant defence systems consisting of enzymes such as catalase, superoxide dismutase and glutathione peroxidase, as well as non-enzymatic antioxidants such as vitamins (e.g., ascorbic acid, tocopherol), glutathione and flavonoids.

Recently, several reports have suggested that equol has the greatest bioactivity of all isoflavones tested when measured in vitro (7,8). The biological effects of equol, the major metabolite of daidzein, are not as well understood as those of daidzein itself. In our previous study in vitro (9), equol induced cell cycle arrest at the G1/S transition or G/M phase in human breast cancer cells, and this cell cycle arrest led to apoptosis induction. This finding is consistent with previous studies indicating that daidzein may be an effective agent against cell growth in some cancer cell types (10,11). Thus, equol apparently has anticancer effects similar to its pro-drug daidzein. However, the equol anticancer effects and its mechanisms are not as well understood as those of daidzein in vivo.

Therefore, we investigated the anticancer effects of equol in an animal model using a chemical carcinogen. Moreover, the mechanism of the anticancer effects of equol via antioxidation and the induction of apoptosis was explored. First, the
proapoptotic effect of equol was investigated in a well-defined rat mammary gland tumor model. To induce mammary carcinoma in rats, we used polycyclic aromatic hydrocarbon 7,12-dimethylbenz(a)anthracene (7,12-DMBA), a well-known chemical carcinogen that produces mammary tumors that are morphologically and histologically similar to those found in humans. A single dose of 100 mg/kg DMBA in SD rats will produce a mammary carcinoma yield of 100% roughly 8 weeks post-treatment (12). Second, the effects of equol in modulating the cancerous response to oxidative stress was investigated in the livers of mice exposed to DMBA for 1 week after equol pretreatment. 7,12-DMBA also acts as an indirect carcinogen. Although the liver is not a target of DMBA-induced carcinogenesis, DMBA is first metabolized by cytochrome P_{450} to form carcinogenic metabolites such as diol epoxides that can be transported to the mammary glands, resulting in DMBA-DNA adducts (13,14). In addition, DMBA induces substantial oxidative damage resulting in the formation of ROS such as peroxides, hydroxyl and superoxide anion radicals in organs such as the liver and mammary glands (15-17). This study was designed to evaluate the potential benefit of equol as a chemopreventive and/or chemotherapeutic agent for human cancer cells.

Materials and methods

Equol administration and sample preparation. All animals (Central Lab. Animal Inc., Seoul, Korea) were acclimated for 1 week under standard environmental condition with AIN 93M diet (Dyets, Bethlehem, PA, USA) and allowed free access to water. The entire experiment consisted of two parts during 8 weeks. In the first, Sprague-Dawley rats were randomly divided into three groups, each comprising of eight animals. Equol (LC Laboratories®, MA, USA) was dissolved in water and administered orally to rats at a dose of 5 and 25 mg/kg BW for 8 weeks after a single dose of DMBA (100 mg/kg). As controls, rats were divided into vehicle alone and DMBA alone groups. In the second part, ICR mice were orally administered equol daily at a dose of 5 and 25 mg/kg BW for 7 weeks before a single dose of DMBA (34 mg/kg/week). After equol administration animals were followed for 1 week continuously. The control groups were the same as above and each group were comprised of six mice. The period and dosage of the DMBA treatments are thought to increase the incidence of cancer and cancer development. DMBA (Sigma) was dissolved in corn oil and was also administered orally to all animals. 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.

At the end of the experiment, all animals were rapidly anesthetized using ether at 6 h after final administration of equol. Their livers and mammary gland tumors were isolated, blotted, weighed, frozen in liquid nitrogen and stored at -70°C until assayed. Mammary gland tumors volume was measured using calipers and calculated as following formula: \( V = \frac{4}{3} \pi r^3 \). For assay, each livers from mice were homogenized for ~45 sec in 9 volume of ice-cold 10 mM phosphate-buffered saline (PBS, pH 7.4) containing 1.15% KCl. Homogenates for oxidative stress biomarker (TBARS and carbonyl content) measurement were 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 post-mitochondrial fraction transferred for GSH-related analysis. To obtain the cytosolic fractions for 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 L.90 ultracentrifuge. Post-mitochondrial and cytosolic fractions were stored at -80°C in aliquots until analysis within one week.

Immunoblotting assay. Liver samples were homogenizing with in RIPA (radio-immunoprecipitation assay) buffer (1% nonidet P-40, 150 mM sodium chloride, 0.05% deoxycholic acid, 1% sodium dodecyl sulfate, 50 mM Tris, pH 7.5) containing protease inhibitor and lysed for 1 h at 4°C. The supernatant was separated by centrifugation, and protein concentration was determined by Bradford protein assay kit II (Bio-Rad Laboratories, CA, USA). Proteins (50 µg/well) denatured with sample buffer were separated by 10% SDS-polyacrylamide gel. 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 PCNA, p21Cip1, p53, Bcl-2, Bax, cytochrome c, cleaved caspase-3 (19 kDa), cleaved PARP 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 h at room temperature. The specific protein bands were detected by Opti-4CN Substrate kit (Bio-Rad).

Oxidative stress measurement. TBARS (thiobarbituric acid reactive substances) value was determined by measuring the concentration of malondialdehyde according to the method of Ohkawa et al (18) and its calculated according to the molar absorption coefficient of MDA (malondialdehyde), \( \varepsilon = 1.56 \times 10^5 \text{M}^{-1} \text{cm}^{-1} \) at 535 nm. The formation of protein carbonyl in the liver was determined using DNPH (dinitrophenyl hydrazine) according to the method of Reznick and Packer (19). The absorbance was read at 365 nm and the results were expressed as moles of DNPH incorporated/100 mg protein using a molar extinction coefficient of 21 M^{-1} cm^{-1}.

Antioxidant enzyme activities. Superoxide dismutase activity was assayed according to the pyrogallol autoxidation method of Marklund and Marklund (20). Each unit of SOD activity was defined as the quantity of enzyme that inhibited auto-oxidation of pyrogallol by 50% under experimental conditions. Catalase activity was assayed by the method of Aebi (21). Catalase activity was calculated as nmol of H_{2}O_{2} decomposed/ min/mg protein. Activities of glutathione peroxidase and reductase were determined with a spectrophotometer by measuring the disappearance of NADPH at 340 nm, based on the methods of Flohe and Gunzler (22) and Carlberg and Mannervik (23), respectively. The enzyme activities of glutathione peroxidase and reductase was defined as nmol NADPH oxidized/min/mg protein using molar extinction coefficient of 6.22x10^{-5} M^{-1}cm^{-1}. Protein concentration was determined by Bradford protein assay kit II (Bio-Rad).
**Determination of total GSH and GSSG.** The concentration of glutathione was measured with use of o-phthalaldehyde as a fluorescent reagent according to method of Hissin and Hilf (24). For GSSG measurement, each sample was incubated with N-ethyl maleimide to interact with GSH present in the tissue homogenate. The content of GSH and GSSG (µg/mg protein) was obtained from a standard curve and then the GSH/GSSG ratio was calculated.

**DNA fragmentation assay.** Liver samples were homogenized in lysis buffer (20 mM EDTA, 0.5% CHAPS, 100 mM Tris, pH 8.0) and incubated at 37°C with RNase A at a concentration of 200 µg/ml for 1 h, followed by treatment overnight with proteinase K at 55°C. DNA was extracted from 25,000 x g centrifugation fraction using phenol-chloroform method as follows: a phenol:chloroform:isoamyl alcohol mixture (25:24:1) was used to remove the proteins. After protein removal, the suspension was incubated with 100% cold ethanol containing 0.1 M sodium acetate for 24 h. The precipitated DNA was washed with 70% ethanol, re-suspended, run on 1.5% agarose gel at 70 V for 1.5 h and then visualized under UV light.

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

**Results**

**Effects of equol on body weight and tumor weight in rats with DMBA-induced mammary gland tumors.** In this study, a significant decrease in body weight of ~15% was observed in the DMBA-treated group, as compared with the control group (P<0.05, Fig. 1). Equol administration did not affect the recovery of body weight. However, equol reduced the tumor burden significantly, as evidenced by tumor weight decreases of 18.44 and 28.38% at 5 and 25 mg/kg BW, respectively, compared with the DMBA group (P<0.05). Similarly, equol reduced the tumor volume significantly in a dose-dependent manner.

**Effects of equol on the expression of PCNA in rats with DMBA-induced mammary gland tumors.** Moreover, the expression of PCNA, a nuclear protein that is present during S phase, was used as a marker of cellular proliferation in the mammary glands of DMBA-treated rats. PCNA expression has also been used to measure the proliferation rate of tumors. In the mammary gland tumors of rats treated with DMBA, PCNA expression was increased 1.98-fold, as compared with control rats (P<0.05, Fig. 2A). The overexpression was reduced by 26.77 and 36.87% in the groups given 5 and 25 mg equol/kg BW, respectively, as compared with the mammary gland tumors of rats treated with DMBA.

**Pro-apoptotic effects of equol on DMBA-induced mammary gland tumor cells.** Equol administration significantly increased p21 protein by 29.75 and 78.50% at 5 and 25 mg/kg BW, respectively, although there were slight increases in the mammary gland tumors of rats treated with DMBA, as compared with the control group (Fig. 2B). The p53 protein was also increased (13.25%) slightly in the mammary gland tumors of rats treated with DMBA, as compared with the control group. However, p53 expression was significantly activated by 1.55 and 3.10% at 5 and 25 mg equol/kg BW, respectively, as compared with the mammary gland tumors of rats treated with DMBA.

In addition, Bcl-2 expression as an anti-apoptotic factor increased 2.45-fold, as compared with the control group. Equol administration reduced these changes in a dose-dependent manner (decreases of 24.62 and 38.01% at 5 and 25 mg/kg BW, respectively, P<0.05, Fig. 3). Bax expression was significantly elevated by approximately 2-fold in both equol groups, regardless of the dose. Similarly, equol increased cleaved caspase-3 and PARP expression in a dose-dependent manner, as compared with the mammary gland tumors of rats treated with DMBA. At a higher dose of equol, cleaved caspase-3 and PARP expression were increased by 90.39 and 86.12%, respectively, as compared with mammary gland tumors of rats treated with DMBA.

**Effects of equol on the oxidative damage in mice treated with DMBA.** DMBA treatment of mice caused dramatic increases in their levels of oxidative stress biomarkers such as serum 8-hydroxy-2-deoxyguanosine (8-OHdG), hepatic carbonyls and thiobarbituric acid-reactive substances (TBARS), compared with the levels in control group (1.43-, 1.54- and 3.59-fold, respectively, compared to the control, P<0.05, Fig. 4). Pre-administration of equol at 5 or 25 mg/kg body weight partially reversed this increase in serum 8-OHdG and hepatic carbonyls levels. In DMBA group, pre-administration of equol at 25 mg/kg caused levels of 8-OHdG and hepatic carbonyls to decrease by 22.99 and 23.83%, respectively, compared with non-pre-treated mice. Moreover, pre-administration of equol...
at 5 or 25 mg/kg significantly decreased the TBARS level by 35.97 and 64.01%, respectively, compared with the DMBA group.

Effects of equol on the antioxidant defense enzymes in mice treated with DMBA. Catalase activity and total superoxide dismutase (SOD) activity were significantly lower by 48.31 and 63.08%, respectively, in mice treated with DMBA, compared with non-treated (control) mice (P<0.05, Fig. 5A). Pre-administration of equol at 5 or 25 mg/kg significantly increased catalase activity by 1.29- and 1.83-fold, respectively. Total SOD activity in the DMBA and control groups was 0.41 and 0.65 U/mg protein, respectively. Pre-administration of equol at 5 or 25 mg/kg significantly
equol at 5 or 25 mg/kg caused total SOD activity to increase in a dose-dependent manner, although this increase was significant only at the higher dose. Cu/Zn- and Mn-SOD expression followed a similar pattern (Fig. 5B).

**Effects of equol on the GSH-related system in mice treated with DMBA.** The ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) was significantly lower (by 19.6%) in DMBA-treated mice than in the controls (Fig. 6A). Equol pre-administration increased the GSH/GSSG ratio in a dose-dependent manner, although the change was not significant. The basal glutathione peroxidase and glutathione reductase activities were 30.56 and 27.41% lower, respectively, in DMBA-treated mice than in the non-treated controls (Fig. 6B). These activities were increased by equol pre-administration in a dose-dependent manner; at the higher dose (25 mg/kg), the difference was significant (25.36 and 22.81% increase for GSH-px and GR, respectively, compared with the value in DMBA group).

**Anti-apoptotic effects of equol in mice treated with DMBA.** Marked DNA fragmentation was observed in the cells of DMBA-treated mice (Fig. 7A). Equol pre-treatment efficiently prevented this fragmentation in a dose-dependent manner. We confirmed that DMBA causes apoptosis by measuring the expression of apoptosis-related proteins in DMBA group (Fig. 7B and C). In these mice, Bcl-2 expression decreased and Bax expression increased. Levels of cleaved caspase-3 and poly(ADP-ribose) polymerase (PARP) also increased in DMBA group. These apoptotic changes were reversed by pre-administration of equol at either dose.

**Discussion**

In the present study, most of all, equol reduced tumor formation as well as PCNA expression in rats with mammary gland tumors induced by DMBA. Cellular proliferation is an integral part of carcinogenesis, with important roles in several steps of the carcinogenic process, including initiation, promotion and progression (25,26). This is consistent with previous reports that showed PCNA overexpression in a wide range of human tumors (27,28), as well as in DMBA-induced mammary gland tumors (29,30). Recently, isoflavones have been found to act as general cell growth inhibitors and apoptosis inducers, which makes them prime candidates in the development of new anticancer compounds (2). In addition, several studies have shown that isoflavones protect against DMBA-induced rat mammary tumorigenesis (31,32). Thus, the proapoptotic capacity of equol as a major metabolite of the isoflavone
daidzein in mammary gland tumors may be valuable in the development of new anticancer drugs.

Previously we reported that equol induced apoptosis through the p53-dependent pathway in human breast cancer cell lines (9). The tumor suppressor gene p53 is regarded as a key factor in the balance between cell survival and death, via regulation of both the G1 and G2/M portions of the cell cycle (33). The activation of p53 in response to DNA damage leads to cell cycle arrest and inhibition of cell proliferation (34,35). It has been reported that the stimulation of p53 expression by chemopreventive agents causes DNA damage and activates p21\textsuperscript{Cip1} by interacting with its regulatory elements directly; this p21\textsuperscript{Cip1} protein occurs in cells undergoing either p53-associated G1 arrest or apoptosis (36).

Equol administration significantly increased p21\textsuperscript{Cip1} and p53 by equol administration in DMBA-induced mammary gland tumors of rats could be responsible for the tumor growth inhibitory effects of equol through apoptosis induction and cell cycle arrest. Therefore, the upregulation of p21\textsuperscript{Cip1} and p53 by equol administration in DMBA-induced mammary gland tumors of rats could be responsible for the tumor growth inhibitory effects of equol through apoptosis induction and cell cycle arrest.

Furthermore, equol administration induced apoptosis in DMBA-induced mammary gland tumors of rats. Bcl-2 family proteins are critical regulators of the apoptotic pathway and control both mitochondrial permeability and cytochrome c expression (37,38). The Bcl-2 family contains several major antiapoptotic members, including Bcl-x(L) and Bcl-2, as well as the major proapoptotic proteins Bax and Bak. It has been reported that Bcl-2 overexpression inhibits the transcriptional activation of Bax (39). Overexpression of antiapoptotic proteins such as Bcl-2 may contribute to tumor formation in DMBA-treated SD rats. Equol administration significantly induced apoptosis in the mammary gland tumors by increasing Bax expression while decreasing that of Bcl-2, with a subsequent increase in the expression of cytochrome c. The release of cytochrome c from mitochondria to the cytoplasm is a key step in the initiation of apoptosis. In addition, equol administration significantly restored the reduction of cleaved caspase-3 expression by DMBA.

Caspases, which comprise a family of cysteine proteases, are the central mediators of programmed cell death. Caspase-3 is a frequently activated death protease that catalyzes the specific cleavage of many key cellular proteins (40,41). PARP is another characteristic hallmark of apoptosis, and PARP expression was induced by equol in a dose-dependent manner.

In the second part, we observed the effect of equol administration on mice experiencing DMBA-induced oxidative stress. DMBA induces substantial oxidative damage to liver and mammary glands (15,16) which are closely associated with tumorigenesis. To evaluate the oxidative stress induced by DMBA and the antioxidant effect of equol on this damage, we measured levels of serum 8-OHdG (oxidized DNA bases), carbonyls and TBARS to assess DNA oxidation, protein oxidation and lipid peroxidation, respectively. Although the mechanisms linking oxidative stress to numerous pathologies have not yet been definitively recognized, proteins can be damaged by oxygen radicals, leading to the loss of enzymatic activity and the conversion of amino acids to carbonyl derivatives (42), and oxidatively modified DNA may also play a role in human carcinogenesis (43). Oxidative stress produces deleterious effects by initiating lipid peroxidation directly or by acting as second messengers for the primary free radicals that initiate lipid peroxidation (44).

Lipid peroxidation and protein oxidation were significantly higher in DMBA group than in control group. This is consistent with previous reports that DMBA induces critical oxidative damage in the liver \textit{in vivo} (45,46). In a previous study, daidzein was shown to be protective against oxidative stress; it significantly decreased the lipid peroxidation that normally occurs in vitamin E-deprived rats. In the present study, equol at all doses tested significantly inhibited oxidative damage, as shown by the decrease in levels of oxidative stress biomarkers induced by DMBA, such as serum 8-OHdG, carbonyls and
flavonoids, which act as general cell growth inhibitors and is a promising target for cancer chemotherapy (52,53), and cleaved caspase-3 and PARP to their normal levels. Apoptosis in a dose-dependent manner, and it restored Bcl-2, Bax, administration reduced DMBA-induced DNA fragmentation (51). Consistent with these results, we found that DMBA treatment (80 mg/kg) in the adrenal cortices of the mice similarly, apoptosis was detected 24 and 36 h after DMBA
Swiss albino mice treated with DMBA exhibited increased DNA fragmentation and apoptosis has been reported (50). This is consistent with previously reported data showing that antioxidant intake contributes to a lower incidence of various cancers. Thus, as an antioxidant, equol might be useful as a nutritional supplement or as a basis for pharmaceutical drugs such as chemotherapeutic agents.

In the present study, the changes induced by DMBA led to the induction of apoptosis. DNA fragmentation was observed in the livers of mice treated with DMBA. DNA fragmentation is a hallmark of apoptosis and a positive association between DNA fragmentation and apoptosis has been reported (50). This is consistent with previously reported data showing that Swiss albino mice treated with DMBA exhibited increased levels of apoptosis in the liver within 8 days of treatment (17); similarly, apoptosis was detected 24 and 36 h after DMBA treatment (80 mg/kg) in the adrenal cortices of the mice (51). Consistent with these results, we found that DMBA significantly decreased expression of Bcl-2 and increased levels of Bax, cleaved caspase-3 and PARP expression. Equol administration reduced DMBA-induced DNA fragmentation in a dose-dependent manner, and it restored Bcl-2, Bax, cleaved caspase-3 and PARP to their normal levels. Apoptosis is a promising target for cancer chemotherapy (52,53), and flavonoids, which act as general cell growth inhibitors and

apoptosis inducers, are prime candidates in the development of new anticancer compounds (54-56). However, under pathophysiological conditions, apoptosis can induce inflammation due to the onset of secondary necrosis (57,58). Thus, equol may modulate apoptosis-related gene expression in mice with DMBA-induced apoptosis as a result of a protective mechanism in the liver.

Taken together, equol administration reduced tumor formation in DMBA-induced mammary gland tumors of rats via multiple pathways by which equol administration results in apoptosis induction via cell cycle arrest by the upregulation of p21Waf1 and p53. Additionally, this study was supported by the finding that equol shows anticancer activity via an antiproliferative effect and cell cycle arrest in vitro. Moreover, these results suggest a hepatoprotective function for equol that occurs via an anti-apoptotic effect, making equol an attractive candidate for use in anticancer medicine for chemotherapeutics.

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References


