Equol, a Metabolite of the Soybean Isoflavone Daidzein, Inhibits Neoplastic Cell Transformation by Targeting the MEK/ERK/p90RSK/Activator Protein-1 Pathway

Received for publication, February 20, 2007, and in revised form, July 11, 2007. Published, JBC Papers in Press, August 27, 2007, DOI 10.1074/jbc.M701459200

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Daidzein and genistein are isoflavones found in soybean. Genistein is known to exhibit anticarcinogenic activities and inhibit tyrosine kinase activity. However, the underlying molecular mechanisms of the chemopreventive activities of daidzein and its metabolite, equol, are not understood. Here we report that equol inhibits 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced neoplastic transformation of JB6 P+ mouse epidermal cells by targeting the MEK/ERK/p90RSK/activator protein-1 signaling pathway. TPA-induced neoplastic cell transformation was inhibited by equol, but not daidzein, at noncytotoxic concentrations in a dose-dependent manner. Equol dose-dependently attenuated TPA-induced activation of activator protein-1 and c-fos, whereas daidzein did not exert any effect when tested at the same concentrations. The TPA-induced phosphorylation of ERK1/2, p90RSK, and Elk, but not MEK or c-Jun N-terminal kinase, was inhibited by equol but not by daidzein. In vitro kinase assays revealed that equol greatly inhibited MEK1, but not Raf1, kinase activity, and an ex vivo kinase assay also demonstrated that equol suppressed TPA-induced MEK1 kinase activity in JB6 P+ cell lysates. Equol dose-dependently inhibited neoplastic transformation of JB6 P+ cells induced by epidermal growth factor or H-Ras. Both in vitro and ex vivo pull-down assays revealed that equol directly bound with glutathione S-transferase-MEK1 to inhibit MEK1 activity without competing with ATP. These results suggested that the antitumor-promoting effect of equol is due to the inhibition of cell transformation mainly by targeting a MEK signaling pathway. These findings are the first to reveal a molecular basis for the anticancer action of equol and may partially account for the reported chemopreventive effects of soybean.

Carcinogenesis is characterized as a multistage process that includes initiation, promotion, and progression stages. Cancer prevention strategies that involve intervention at the tumor promotion stage, a reversible and long-term process, seem to be more practical than those intervening at the tumor initiation stage, which is an irreversible and short-term process. Activator protein-1 (AP-1) is a well characterized transcription factor composed of homodimers and/or heterodimers of the Fos and Jun protein families and plays a key role in “preneoplastic-to-neoplastic” transformation and proliferation (1). A diverse variety of stimuli induce AP-1 binding to various genes that govern cellular processes such as transformation and proliferation (2). In particular, 12-O-tetradecanoylphorbol-13-acetate (TPA), H-Ras, and epidermal growth factor (EGF) are the most common experimental stimuli used to activate AP-1 and induce cellular transformation in many different cell types and animal models (3).

Many mechanisms are involved in the up- and down-regulation of AP-1 activity (2). The mitogen-activated protein (MAP) kinases are the most common signaling pathways known to mediate AP-1 function (4), and the blocking of MAP kinases leads to the inhibition of AP-1 transactivation and subsequent cell transformation (5). These kinase families include extracellular-signal-regulated protein kinases (ERKs), c-Jun N-terminal kinases, and the p38 family of kinases. Although the proteins have some redundancy in function, ERKs generally play a critical role in transmitting signals initiated by tumor promoters such as TPA, EGF, and platelet-derived growth factor (6, 7). MEK is a dual-specificity protein kinase that phosphorylates its downstream target ERK on specific tyrosine and threonine residues. The constitutive activation of MEK1 results in cellular transformation, whereas a small molecular inhibitor of MEK was shown to suppress transformation and tumor growth in both cell culture and mouse models (6, 8). Moreover, a mutant H-ras gene perpetually activates the MEK/ERK signaling pathway and drives cells to develop a more aggressive cancer-like

‡ The work is supported in part by The Hormel Foundation and National Institutes of Health Grants CA120388, CA111536, CA88961, and CA81064 and by BioGreen21 Program Grant 20070301-034-042, Rural Development Administration, Republic of Korea. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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5 The abbreviations used are: AP-1, activator protein-1; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase; TPA, 12-O-tetradecanoylphorbol-13-acetate; EGF, epidermal growth factor; FBS, fetal bovine serum; MOPS, 4-morpholinepropanesulfonic acid; ROS, reactive oxygen species; GST, glutathione S-transferase; MEM, Eagle’s minimum essential medium; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxy-phenyl)-2-(4-sulfonfonyl)-2H-tetrazolium.
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Naturally occurring dietary phenolic phytochemicals originating from these plant-based foods have been suggested to have chemopreventive effects in carcinogenesis, particularly in the stage of promotion (7, 14, 15). Soybean is an excellent source of dietary phenolic substances in the Asian diet (13, 16). The two major isoflavones in soybean are genistein and daidzein (see Fig. 1A), which together compose 100–300 mg/100 g of soybean and are found in tissues as glycoside conjugates (17). These compounds are heat-stable and, hence, are also abundant in many processed and fermented soybean products (18).

Genistein was originally described as a protein-tyrosine kinase inhibitor (19), and it also has been reported to suppress angiogenesis (20) and cell cycle progression (21) and stimulate apoptosis (22). Daidzein is another prominent isoflavone found in soybean foods but has consistently been reported to be less active than genistein (23). This conclusion is probably not accurate because, unlike genistein, daidzein is converted to equol (Fig. 1B) by the intestinal microflora, which can lead to bioactivation (24). Specifically, after ingestion, daidzein is converted to equol (70%), dihydrodaidzein (10–25%), and O-desmethylangolensin (5–20%) (25). The half-life in the body for equol is significantly longer compared with daidzein or genistein (26), and equol is consistently reported to be present at high levels in the blood (27). Several independent lines of evidence indicate that equol is one of the most biologically active metabolites of daidzein.

In many cell types equol is more potent than daidzein in terms of its antioxidant properties (28). For example, equol at concentrations within the physiological range were shown to protect against hydrogen peroxide-mediated DNA damage in human lymphocytes as determined by alkaline single-cell gel electrophoresis (29). Equol was reported to exhibit protective effects against UV-induced DNA damage in hairless mice (30). Moreover, equol was shown to protect against UV-induced skin cancer in the hairless mouse model (31). The UV-induced activation of ornithine decarboxylase, a skin tumor promotion biomarker enzyme, was attenuated by equol treatment, indicating that the anticancer activity of equol may be attributed to its inhibition of the tumor promotion phase of carcinogenesis (31). These accumulated data represent evidence suggesting that equol is a potent chemopreventive agent against carcinogenesis, particularly skin cancer, but the underlying molecular mechanisms and molecular target(s) remain unclear.

The skin is highly sensitive to TPA induction of MEK/ERK/AP-1 signaling activation and tumorigenesis (32). In JB6 mouse epidermal cell lines, TPA and EGF were shown to induce AP-1 transcriptional activity in promotion-sensitive (P+), but not in promotion-resistant (P−), cellular phenotypes (1). When AP-1 induction was blocked, P+ cells reverted to the P− phenotype, indicating that AP-1 activity is required for TPA- or EGF-induced cell transformation (1). The JB6 P+ mouse epidermal cell line provides a validated model for screening cancer chemopreventive agents and elucidating their molecular mechanisms of action (33). In the present study we compared the effects of equol and daidzein on TPA-induced AP-1 activity and cell transformation in JB6 P+ cells. We found that equol, but not daidzein, was a potent inhibitor of MEK activity and subsequently inhibited AP-1 transactivation and cell transformation. The results of this investigation suggested a molecular mechanism that underlies the chemopreventive activity of equol and might partially account for the chemopreventive effects of soybean foods.

EXPERIMENTAL PROCEDURES

Chemicals—Equol, daidzein, TPA, and trypan blue solution were obtained from Sigma. Eagle's minimum essential medium (MEM), basal medium Eagle, gentamicin, and i-glutamine were purchased from Invitrogen; fetal bovine serum (FBS) was purchased from Gemini Bio-Products (Calabasas, CA). PD098059 was purchased from Calbiochem. The antibodies against phosphorylated MEK (Ser-217/221), phosphorylated ERK (Tyr-202/Tyr-204), total ERK, phosphorylated c-Jun N-terminal kinase (Thr-183/Tyr-185), total c-Jun N-terminal kinase, phosphorylated p90RSK (Thr-359/Ser-363), total p90RSK, phosphorylated Elk-1 (Ser-383), and total Elk-1 were purchased from Cell Signal Biotechnology (Beverly, MA). The antibody against total MEK was from Santa Cruz Biotechnology (Santa Cruz, CA). The MEK1 and Raf1 kinase assay kit was obtained from Upstate Biotechnology (Lake Placid, NY). CNBr-Sepharose 4B, glutathione-Sepharose 4B, and [γ-32P]ATP were purchased from Amersham Biosciences, and the protein assay kit was from BioRad. G418, the CellTiter96 Aqueous One Solution Cell Proliferation Assay kit, and the luciferase assay substrate were purchased from Promega (Madison, WI).

Cell Culture—The JB6 P+ mouse epidermal (JB6 P+) cell line and H-Ras-transformed JB6 P+ mouse epidermal (H-Ras JB6 P+) cell line were cultured in monolayers at 37 °C in a 5% CO2 incubator in MEM containing 5% FBS, 2 mM l-glutamine, and 25 μg/ml gentamicin. The JB6 mouse epidermal cell line was stably transfected with an AP-1 luciferase reporter plasmid and maintained in MEM supplemented with 5% FBS containing 200 μg/ml G418.

Proliferation Assay—To estimate cell proliferation, JB6 P+ cells were seeded (103 cells/well) in 96-well plates with 5% FBS, MEM at 37 °C in a 5% CO2 incubator. After culturing for the indicated times, 20 μl of CellTiter G418 Aqueous One Solution (Promega) were added to each well, and the cells were then incubated for 1 h at 37 °C in a 5% CO2 incubator. Absorbance was measured at 492 and 690 nm.

Viability Assay—The trypan blue exclusion assay was based on the capability of viable cells to exclude the dye. Because viable JB6 P+ cells maintain membrane integrity, the cells do not allow trypan blue dye to pass through the cell membrane. Cells with damaged membrane appeared blue due to their accumulation of dye and were counted as dead. Trypan blue (0.4%) was added to JB6 P+ cells, and after 5 min, cells were loaded into a hemocytometer and counted. The number...
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of viable cells was calculated as percent of the total cell population.

Anchorage-independent Transformation Assay—The effects of daidzein or equol on TPA-induced cell transformation were investigated in JB6 P+ cells. Cells (8 × 10^5/ml) were exposed to TPA with or without daidzein (0–20 μM) or equol (0–100 μM) in 1 ml of 0.33% basal medium Eagle agar containing 10% FBS or in 3.5 ml of 0.5% basal medium Eagle agar containing 10% FBS. The cultures were maintained at 37 °C in a 5% CO2 incubator for 14 days, and the cell colonies were counted under a microscope with the aid of the Image-Pro Plus software (Version.4) program (Media Cybernetics, Silver Spring, MD) as described by Colburn et al. (34).

Luciferase Assay for AP-1 Transactivation—Confluent monolayers of JB6 P+ cells stably transfected with an AP-1 luciferase reporter plasmid were trypsinized, and 8 × 10^5 viable cells suspended in 100 μl of 5% FBS, MEM were added to each well of a 96-well plate. Plates were incubated at 37 °C in a humidified atmosphere of 5% CO2. When cells reached 80–90% confluence, they were starved by culturing them in 0.1% FBS, MEM for another 24 h. The cells were then treated for 1 h with daidzein (0–100 μM) or equol (0–100 μM) and then exposed to 20 ng/ml TPA for 24 h. After treatment, cells were disrupted with 100 μl of lysis buffer (0.1 M potassium phosphate buffer (pH 7.8), 1% Triton X-100, 1 mM dithiothreitol, and 2 mM EDTA), and the luciferase activity was measured using a luminometer (Luminoskan Ascent, Thermo Electron, Helsinki, Finland).

Reporter-gene Assay—The reporter gene assay for firefly luciferase activity was performed using lysates from transfected cells. In addition, the reporter gene vector pRL-SV40 (Promega) was co-transfected into each cell line, with the transfection efficiencies normalized to the Renilla luciferase activity generated by this vector. Cell lysates were prepared by first washing the transfected JB6 P+ cells once in phosphate-buffered saline completely, 500 μl of passive lysis buffer (Promega Dual Luciferase Reporter Assay system) were added, and the cells were incubated for 1 h with gentle shaking. The lysate was then transferred to a reaction tube, and the cellular debris was removed by centrifugation. The supernatant fraction was used for the measurement of firefly and Renilla luciferase activities. Cell lysates (20 μl each) were mixed with 100 μl of Luciferase II reagent, and the emitted firefly luciferase light was measured (Luminoskan Ascent). Subsequently, the coelenterazine reagent (100 μl) containing the substrate for the emission of Renilla luciferase light was mixed to normalize the firefly luciferase data. The c-fos luciferase promoter (pFos-WT GL3) and constructs were kindly provided by Dr. Ron Prywes (Columbia University, New York, NY).

Western Blotting—After the cells (1.5 × 10^6) were cultured in a 10-cm dish for 48 h, they were starved in serum-free medium for another 24 h to eliminate the influence of FBS on the activation of mitogen-activated protein kinases. The cells were then treated with daidzein (0–100 μM) or equol (0–100 μM) for 1 h before they were exposed to 20 ng/ml TPA for different time periods. The harvested cells were disrupted, and the supernatant fractions were boiled for 5 min. The protein concentration was determined using a dye-binding protein assay kit (Bio-Rad) as described in the manufacturer’s manual. Lysate protein (20 μg) was subjected to 10% SDS-PAGE and electrophoretically transferred to a polyvinylidene difluoride membrane (Amersham Biosciences). After blotting, the membrane was incubated with the specific primary antibody at 4 °C overnight. Protein bands were visualized by the chemiluminescence detection kit (Amersham Biosciences) after hybridization with the horse-radish peroxidase-conjugated secondary antibody. The relative amounts of proteins associated with specific antibodies were quantified using Sicon Image (NIH, Bethesda, MD).

In Vitro MEK1 and Raf1 Kinase Assay—The in vitro kinase assays were performed in accordance with the instructions provided by Upstate Biotechnology. In brief, every reaction contained 20 μl of assay dilution buffer (20 mM MOPS (pH 7.2), 25 mM β-glycerolphosphate, 5 mM EGTA, 1 mM sodium orthovanadate, and 1 mM dithiothreitol) and a magnesium-ATP mixture buffer. For MEK1, 1 μg of the inactive ERK2 substrate peptide was also included. For Raf1, 0.4 μg of inactive MEK1 and 1 μg of inactive ERK2 were included. A 4-μl aliquot was removed from the reaction mixture containing 20 μg of myelin basic protein substrate peptide and 10 μl of diluted [γ-32P]ATP solution and incubated at 30 °C for 30 min. This mixture was incubated for 10 min at 30 °C, and then 25-μl aliquots were transferred onto p81 paper and washed 3 times with 0.75% phosphoric acid for 5 min per wash and once with acetone for 2 min. The radioactive incorporation was determined using a scintillation counter. The effects of daidzein (0–100 μM) or equol (0–100 μM) were evaluated by separately incubating each compound with the reaction mixtures at 30 °C for 30 min. Each experiment was performed 3 times.

Ex Vivo MEK1 Immunoprecipitation and Kinase Assay—JB6 P+ cells were cultured to 80% confluence and then serum-starved in 0.1% FBS, MEM for 24 h at 37 °C. Cells were either treated or not treated with daidzein (0–100 μM) or equol (0–100 μM) for 1 h, then treated with 20 ng/ml TPA for 30 min, disrupted with lysis buffer (20 mM Tris/HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl, 1 mM EGTA, 1% Triton X-100, 1 mM β-glycerophosphate, 1 mg/ml leupeptin, 1 mM Na3VO4, and 1 mM phenylmethylsulfonyl fluoride), and finally centrifuged at 14,000 rpm for 10 min in a microcentrifuge. The lysates containing 500 μg of protein were used for immunoprecipitation with an antibody against MEK1 and then incubated at 4 °C overnight. Protein A/G Plus-agarose beads were then added, and the mixture was continuously rotated for another 3 h at 4 °C. The beads were washed 3 times with kinase buffer (20 mM MOPS (pH 7.2), 25 mM β-glycerolphosphate, 5 mM EGTA, 1 mM sodium orthovanadate, and 1 mM dithiothreitol) and then resuspended in 20 μl of 1× kinase buffer supplemented with 1 μg of inactive ERK2 and incubated for an additional 30 min at 30 °C. Then myelin basic protein (20 μg) and 10 μl of diluted [γ-32P]ATP solution were added, and the mixture was incubated for 10 min at 30 °C. A 20-μl aliquot was transferred onto p81 paper and washed 3 times with 0.75% phosphoric acid for 5 min per wash and once with acetone for 2 min. The radioactive incorporation was determined using a scintillation counter. Each experiment was performed 3 times.
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*In Vitro Pulldown Assay*—Recombinant MEK1 (2 μg), Raf1 (2 μg), or a JB6 P+ cellular supernatant fraction (500 μg) was incubated with equol- or daidzein-Sepharose 4B (or -Sepharose 4B as control) beads (100 μl, 50% slurry) in reaction buffer (50 mM Tris (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1 mM dithiothreitol, 0.01% Nonidet P-40, 2 μg/ml bovine serum albumin, 0.02 mM phenylmethylsulfonyl fluoride, 1 × protease inhibitor mixture). After incubation with gentle rocking overnight at 4 °C, the beads were washed 5 times with buffer (50 mM Tris, pH 7.5), 5 mM EDTA, 150 mM NaCl, 1 mM dithiothreitol, 0.01% Nonidet P-40, 0.02 mM phenylmethylsulfonyl fluoride), and proteins bound to the beads were analyzed by immunoblotting.

**ATP and Equol Competition Assay**—Briefly, 0.2 μg of active MEK1 was incubated with 100 μl of equol-Sepharose 4B or 100 μl of Sepharose 4B in reaction buffer (see “In vitro Pulldown Assay” above) for 12 h at 4 °C, and ATP was added at different concentrations (1, 10, or 100 μM) to a final volume of 500 μl for 30 min. The samples were washed, and then proteins were detected by Western blot.

**Expression and Purification of Recombinant MEK1 Mutants**—For the expression of full-length and deletion mutants of MEK1, the appropriate plasmids (pGEX-MEK1 and deletion mutants for GST-MEK1) were expressed in Escherichia coli BL21. Single colonies were selected after culturing in Luria-Bertani (LB) medium at 37 °C for 16 h with vigorous agitation. When liquid cultures exhibited an absorbance of 0.8 at 600 nm, they were diluted 100-fold with fresh LB medium. Isopropyl-β-D-thiogalactoside was added to these cultures at a final concentration of 0.1 mM, and cultures were then continuously agitated for another 3 h at 25 °C. The bacteria were collected by centrifugation (4000 rpm for 10 min at 4 °C), and the pellets were disrupted by sonication. The lysate was again centrifuged, and the supernatant fraction was saved. A 50% slurry (250 μl) of glutathione-Sepharose 4B beads was added to each sample and then mixed gently for 1 h at room temperature, and GST proteins were purified by immobilized metal affinity chromatography. The molecular mass and relative protein expression were estimated by SDS-PAGE and Coomassie Blue staining.

**Molecular Modeling**—Insight II (Accelrys Inc., San Diego, CA) was used for the docking study and structure analysis with the crystal coordinates of MEK1 (Protein Data Bank code 159J).

**RESULTS**

Equol Has No Cytotoxic Effects on JB6 P+ Cells—Although daidzein has been reported to exhibit antiproliferative activities in several cell types (35), little is known about the effect of equol on cell proliferation. Therefore, we examined the effects of daidzein (Fig. 1A) or equol (Fig. 1B) on the proliferation of JB6 P+ cells using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2H-tetrazolium (MTS) assay. Even though equol is a metabolite of daidzein, it did not significantly affect cell growth at 1, 3, or 5 days after treatment at concentrations up to 100 μM (Fig. 2A). By contrast, daidzein at a concentration of 50 μM was cytotoxic to JB6 P+ cells at 3 or 5 days after treatment (Fig. 2B). These results indicated that, compared with daidzein, equol was less cytotoxic to JB6 P+ cells. To further confirm these results, we examined the effect of equol on the viability of JB6 P+ cells using the trypan blue assay. Results confirmed that equol, at concentrations up to 100 μM, did not significantly affect cell viability at 1, 3, or 5 days after treatment (Fig. 2C), agreeing with the MTS assay results.

Equol, but Not Daidzein, Inhibits TPA-induced Neoplastic Transformation of JB6 P+ Cells—We next examined the effect of equol and daidzein on TPA-induced neoplastic transformation of JB6 P+ cells. Based on the numbers of transformed cell colonies, treatment with equol (Fig. 3A) but not daidzein (Fig. 3B), significantly inhibited TPA-promoted neoplastic transformation of JB6 P+ cells in a dose-dependent manner (Fig. 3A). These results suggested that not only is equol less cytotoxic (Fig. 2) than daidzein, but is more effective at inhibiting TPA-induced neoplastic cell transformation of JB6 P+ cells.

Equol Attenuates TPA-induced AP-1 and c-fos Activation in JB6 P+ Cells—AP-1 activity was previously shown to be required for neoplastic transformation in JB6 P+ cells (1, 36). To determine whether the repression of transformation by equol involves the inhibition of AP-1 activity, we measured AP-1 transactivation using JB6 P+ cells stably transfected with an AP-1 luciferase reporter plasmid. Consistent with the above results for cell transformation, equol inhibited TPA-induced transactivation of AP-1 in a dose-dependent manner (Fig. 4A), whereas daidzein at 25–100 μM exerted no significant effects (Fig. 4B). Because TPA induces c-fos expression mainly through the ERK signaling pathway and subsequently induces AP-1 transactivation, we next investigated whether equol could inhibit c-fos promoter activation using the reporter plasmid carrying the luc gene under the control of the c-fos promoter. The TPA-induced c-fos promoter activity was also suppressed by equol treatment in a dose-dependent manner (Fig. 4C), whereas daidzein exerted no significant effects (Fig. 4D). These results indicated that equol was substantially more effective than daidzein in suppressing AP-1 transactivation in JB6 P+
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Equol Suppresses TPA-induced Phosphorylation of ERK, p90RSK, and Elk-1 but Not MEK or c-Jun N-terminal Kinase, in JB6 P+ Cells—Previous studies have shown that the MEK/ERK signaling pathway is strongly involved in TPA-induced cell transformation and AP-1 transactivation in JB6 P+ cells (1, 36). Thus, we next investigated the effect of equol on the MEK/ERK signaling pathway and found that equol suppressed TPA-induced phosphorylation of ERK, one of its well known substrates, p90RSK, and Elk-1 in JB6 P+/H11001 cells in a dose-dependent manner (Fig. 5A). However, equol had no effect on TPA-induced phosphorylation of MEK in these cells (Fig. 5A). Additionally, equol also had no effect on TPA-induced phosphorylation of c-Jun N-terminal kinase in these cells (data not shown). The TPA-induced activation of the MEK/ERK signaling pathway was inhibited by PD098059, a well known MEK inhibitor that was used as a positive control (Fig. 5C). In contrast, even when used at concentrations up to 100 μM, daidzein had no effect on TPA-induced activation of the MEK/ERK/p90RSK/Elk signaling pathway (Fig. 5B). Overall these results confirmed that equol is markedly more effective than daidzein as a suppressor of the TPA-induced phosphorylation of ERK, Elk-1, and p90RSK.

Equol Specifically Inhibits MEK1 Kinase Activity but Not Raf1 Kinase Activity—We next investigated whether MEK1 might be a molecular target of equol for the inhibition of cell transformation. Results of an in vitro kinase assay indicated that equol, but not daidzein, strongly inhibited MEK1 kinase activity (Fig. 6A). Furthermore, equol, but not daidzein, dose-dependently inhibited TPA-stimulated MEK1 activity in JB6 P+ cell lysates (Fig. 6B). To determine whether equol specifically inhibi-
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In its MEK1 kinase activity, we investigated the effect of equol on the kinase activity of the upstream MEK1 kinase, Raf-1. Equol had no effect on Raf-1 kinase activity in vitro (Fig. 6C). Additionally, overexpression of MEK1 attenuated equol inhibition of TPA-induced AP-1 activation (Fig. 6, D and E). These results indicated that MEK1 is an important target molecule of equol for the inhibition of cell transformation.

Equol Inhibits EGF- or H-Ras-induced Neoplastic Transformation of JB6 P+ Cells—EGF is known to induce cell transformation mainly through the MEK/ERK signaling pathway, and our previous work showed that ERK activity is required for EGF-induced cell transformation in JB6 P+ cells (37). Various human cancers have been found to exhibit mutations and overexpression of ras genes, and one of the most frequent events in carcinogenesis is uncontrolled activation of the Ras signaling pathway (38). An accumulation of data suggests that the upstream effector of the MEK/ERK cascade is Ras/Raf (39). Our previous studies showed that H-Ras activated the growth-signal pathway involving MEK and ERK protein kinases, leading to anchorage-independent growth and elevated AP-1 activity in JB6 P+ cells (10). In the present study we examined the potential inhibitory activities of equol and PD098059 on EGF- or H-Ras-induced cell transformation. Based on the number of cell colonies, equol at concentrations of 50 μM inhibited EGF-induced cell transformation by 41% (Fig. 7A, panel d), and the same concentration also suppressed H-Ras-induced neoplastic transformation of JB6 P+ cells by 49% (Fig. 7B, panel i). We also found that 50 μM PD098059 inhibited EGF- or H-Ras-induced neoplastic transformation of JB6 P+ cells by 79% (Fig. 7A, panel f) and 77% (Fig. 7B, panel k), respectively, suggesting that equol also may inhibit cell transformation by targeting the MEK signaling pathway.

Equol Specifically Binds with MEK1 in Vitro and ex Vivo and Does Not Compete with ATP for Binding with MEK1—The results above indicated that the inhibition of cell transformation by equol involves the inhibition of MEK1 kinase activity but not its upstream phosphorylation. To further confirm whether equol can directly bind with MEK1, we performed an in vitro pulldown assay. MEK1 was found in the equol-Sepharose 4B beads (Fig. 8A, left upper panel) but not in Sepharose 4B beads alone (Fig. 8A, left upper panel). The MEK1 protein was loaded as a control (Fig. 8A, left upper panel, first lane). We also observed en vivo binding of equol and MEK1 in JB6 P+ cell lysates (Fig. 8A, right upper panel). Daidzein did not bind with MEK1 in vitro or ex vivo (Fig. 8A, right or left middle panels). To determine whether equol specifically interacts with MEK1, we investigated in vitro and ex vivo binding of equol and Raf1 (Fig. 8A, lower panels). Results confirmed that equol did not bind with the Raf1 kinase. Therefore, these results indicated that equol has a specificity for MEK1. Furthermore, ATP did not compete with equol for binding with MEK1 (Fig. 8B). These results

FIGURE 4. Effects of equol or daidzein on TPA-induced AP-1 or c-fos activation. A and B, equol, but not daidzein, inhibits TPA-induced AP-1 transactivation. For the luciferase assay, JB6 cells stably transfected with an AP-1 luciferase reporter plasmid were cultured as described under “Experimental Procedures.” The cells were starved in 0.1% FBS, MEM and then treated or not treated with daidzein or equol at the indicated concentrations (25, 50, or 100 μM) for 1 h before they were exposed to 20 ng/ml TPA for 24 h. Luciferase activity was assayed, and AP-1 activity is expressed relative to control cells without TPA treatment. Data are represented as the means ± S.D. of AP-1 luciferase activity calculated from three independent experiments. C and D, equol, but not daidzein, suppresses TPA-induced c-fos promoter activity. For the reporter-gene assay, JB6 P+ cells were transfected with a plasmid mixture containing the c-fos-luciferase reporter gene (0.5 μg) and the pRL-SV40 gene (0.5 μg). At 24 h after transfection, cells were starved for 24 h by incubation in 0.1% FBS, MEM at 37 °C in a 5% CO2 atmosphere. Cells were then treated or not treated with daidzein or equol at the indicated concentrations (25, 50, or 100 μM) for 1 h before they were exposed to 20 ng/ml TPA for 12 h. Firefly luciferase activity was determined in cell lysates and normalized to Renilla luciferase activity, and c-fos-luciferase activity is expressed relative to control cells without TPA treatment. Data are represented as the means ± S.D. of the c-fos-luciferase activity calculated from three independent experiments. The asterisk (*) indicates a significant difference (p < 0.05) between groups treated with TPA and daidzein or equol and the group treated with TPA alone.

FIGURE 5. Effects of equol, daidzein, and PD098059 on TPA-induced phosphorylation of ERK, p90RSK, Elk-1, MEK, and c-Jun N-terminal kinase. A, equol inhibits TPA-induced phosphorylation (p-) of ERK, p90RSK, and Elk-1 but not MEK. B, daidzein did not inhibit TPA-induced phosphorylation of ERK, p90RSK, Elk-1, and MEK. C, PD098059 inhibits TPA-induced phosphorylation of ERK, p90RSK, and Elk-1 but not MEK. Cells were pre-treated with equol (A), daidzein (B), or PD098059 (C) at the indicated concentrations (25, 50, or 100 μM) for 1 h, then stimulated with 20 ng/ml TPA and harvested 15 min later. The levels of phosphorylated and total MEK, ERK, p90RSK, and Elk-1 proteins were determined by Western blot analysis as described under “Experimental Procedures” using specific antibodies against the corresponding phosphorylated and total proteins. Data are representative of two independent experiments that gave similar results.
suggested that equol inhibited MEK1 kinase activity noncompetitively with ATP.

Identification of the Equol Binding Site of MEK1—Previous studies have demonstrated that, like equol, MEK1-selective small molecule inhibitors such as PD098059, PD184352, PD318088, and U0126 do not compete with ATP for binding with MEK1, but at present only the direct binding sites for PD184352 and PD318088 are known (40, 41). To determine the region of binding between equol and MEK1, we constructed three MEK1 deletion mutants from full-length GST-MEK1. One mutant contained only the N-terminal, GST-MEK1 (1–67); one contained the N terminus and the binding region of PD184352, GST-MEK1 (1–206), and one contained the N terminus, the binding region of PD184352, and a phosphorylation site of MEK1, GST-MEK1 (1–225) (Fig. 8C). These deletion mutants were expressed and purified in bacteria as described under “Experimental Procedures.” We then evaluated the binding affinity of the four expressed GST-MEK proteins using the equol-Sepharose 4B pulldown assay. The results indicated that GST-MEK1 (1–206) interacted efficiently with equol, whereas GST-MEK1 (1–67) was not detected (Fig. 8D). These results suggested that equol is a potent inhibitor of MEK1 noncompetitively with ATP and that the region where equol binds with MEK1 might be similar to that of the MEK1 inhibitor PD184351.

DISCUSSION

Dietary phenolic phytochemicals, which are present in fruits, vegetables, grains, and some beverages, have been proposed as primary antioxidant chemopreventive agents (13). However, most free-radical scavengers act in oxidation-reduction reactions, and some phenolic phytochemicals can act as prooxidants and exert toxicity, depending on their structure and the conditions (42–45). In the present study, equol at concentrations from 25 to 100 μM had no effect on cell growth even at 5 days after treatment (Fig. 2A). In contrast, 50 μM daidzein was cytotoxic to JB6 P+ cell growth (Fig. 2B). The higher cytotoxicity of daidzein relative to equol might be attributable to a quinone group of daidzein, which has been suggested to contribute to the production of reactive oxygen species (ROS) in cells. A previous study (46) showed that the presence of the semiquinone radical or benzoquinone resulted in the formation...
of a redox cycle to produce abundant ROS, with the generated ROS exerting cytotoxic effects on JB6 P+ cells. These results support our finding that equol is less cytotoxic to JB6 P+ cells compared with daidzein.

We have compared the effects of nontoxic concentrations of daidzein and equol on TPA-induced neoplastic cell transformation in JB6 P+ cells (Fig. 3). Equol inhibited TPA-induced cell transformation in JB6 P+ cells in a dose-dependent manner from 25 to 100 \( \mu \)M (Fig. 3A), whereas daidzein had no significant effect at these concentrations (Fig. 3B). Although genistein has been shown to induce cell death in many cancer cell types, little is known about its cytotoxic effects on normal non-transformed cells. JB6 P+ cells are considered to be normal cells when not exposed to TPA, EGF, or other tumor promoters. Genistein exerted marked cytotoxicity on JB6 P+ cells in a concentration-dependent manner, with inhibition present even at 5 \( \mu \)M after 1 day (data not shown). Moreover, genistein at nontoxic doses had no effect on cell transformation (data not shown). These results indicated that equol might play a more important role compared with genistein in the chemopreventive activity of soybean products. Equol has been shown to prevent UV-induced DNA damage and activation of ornithine decarboxylase (30, 31). Furthermore, a recent study demonstrated that equol significantly attenuated the portion of tumors progressing from benign papillomas to malignant squamous cell carcinomas (SCC) by 33–58% and reduced the average diameter of SCC by 71–82% (31). Our results support the aforementioned chemopreventive effects of equol against skin carcinogenesis reported for in vivo mouse models.

Previous studies (1, 36) have established the role of AP-1 activation in cellular transformation and tumor promotion in both cell culture and mouse models. When stimulated with the tumor promoter, TPA, AP-1 binds to TPA response elements in the transactivation promoter region and induces transcription of several genes involved in cell proliferation and metastasis. On the other hand, inhibition of AP-1 activity has been shown to lead to suppression of cell transformation (47). All of these results strongly indicated that the inhibition of AP-1 activity leads to the suppression of tumor promotion. Our results indicated that equol inhibited TPA-induced AP-1 and c-fos activation in JB6 P+ cells in a dose-dependent manner, whereas daidzein at 25–100 \( \mu \)M had no effect on AP-1 transactivation (Fig. 4). These results suggested that the inhibitory effects on TPA-induced cell transformation were greater for equol than for daidzein, which can be explained by inhibition of AP-1 activity.

The mitogen-activated protein kinase signaling pathways are critical for AP-1 activation. As indicated earlier, TPA, EGF, and H-Ras induce elevated levels of AP-1 activation and a high frequency of large, tumorigenic, anchorage-independent colony formation of JB6 P+ cells but not of P- cells. The lack of response was shown to be directly attributable to a low level of TPA-, EGF-, or H-Ras-induced phosphorylation of ERK and total ERK protein levels (37). Stable transfection of ERK2 into P- cells restored the response to tumor promoter-induced AP-1 activation and neoplastic cell transformation (37). On the other hand, blocking ERK activity by a dominant negative ERK2 or a MEK1 inhibitor, PD908059, also blocked TPA- or EGF-induced AP-1 and cell transformation (48). These accumulated data demonstrated that MEK/ERK signaling may be used as a target for prevention of carcinogenesis induced by TPA, EGF, or H-Ras. Our results demonstrated that equol, but not daidzein, inhibited the TPA-induced phosphorylation of ERK, p90RSK, and Elk-1, but not MEK, in JB6 P+ cells (Fig. 5). Moreover, equol inhibited MEK kinase activity both in vitro and ex vivo in a dose-dependent manner (Fig. 6). Existing data suggested that most of the relevant mechanisms of cancer prevention attributed to phenolic phytochemicals are not related to their direct antioxidant activity but are due to their ability to directly bind to target molecules, including selected protein kinases, matrix metalloproteinases, and cyclooxygenases (7, 49). Our results suggested MEK as an important molecular target for the antitumor-promoting activity of equol.

As indicated above, the MEK/ERK/p90RSK/AP-1 signaling pathway is believed to be strongly activated and to have a critical role in transmitting signals initiated by TPA, EGF, H-Ras, and platelet-derived growth factor (6, 12). The Ras/MEK/ERK signaling cascade is an essential component of intracellular signaling pathways from activated cell-surface receptors to transcription factors in the nucleus in all metazoan organs. Previous studies have shown that mutation of Ras is present in several
cancer cell lines and that the transforming actions of several oncogenes involve an MEK/ERK/AP-1-dependent signaling pathway (50). In particular, TPA, EGF, and Ras perpetually activate the MEK/ERK signaling pathway and induce a more aggressive cancer-like phenotype in cells, such as anchorage-independent growth and elevated AP-1 activity (11). We found that equol inhibited EGF- and H-Ras-induced neoplastic cell transformation in a dose-dependent manner (Fig. 7). In addition, equol and PD098059 (an inhibitor of MEK) showed similar effects even though slight differences were found in the concentration ranges at which effective inhibition occurred. These results also suggested that equol inhibits cell transformation mainly by targeting the MEK signaling pathway.

We further confirmed by in vitro and ex vivo equol pulldown assays that equol directly binds MEK1 (Fig. 8A) and that equol does not compete with ATP for binding with MEK1 (Fig. 8B), indicating that equol inhibits MEK1 noncompetitively with ATP. Like equol, PD098059, PD184352, PD318088, and U0126 do not compete with ATP in the binding of MEK1, but at present only the direct binding sites of PD184352 and PD318088 are known (40, 41). We found that GST-MEK1 (1–67), containing only the N terminus, was not detected in this assay (Fig. 8D). These results indicated that equol potently inhibited MEK1 without competing with ATP and suggested that the binding region of equol with MEK1 is similar to that of the MEK1 inhibitor PD184351. Future studies should employ x-ray crystallography to elucidate the structure of the equol-MEK1 complex. Future analyses of the molecular mechanisms underlying our observations will help to clarify the antitumor-promoting effects of equol.

Considering the experimental result showing that equol binds to MEK1 without competing with ATP, we carried out a modeling study to investigate the binding of equol to MEK1 (Fig. 9A). Interestingly, equol can be docked to a pocket separate from but adjacent to the ATP binding site similar to PD318088 as observed in the crystal structure of the
Thus, the predicted binding of equol is somewhat similar to that of PD318088. The hydroxyl group at the 7 position of equol can make a hydrogen bond with the backbone carbonyl group of Val-127 in the ATP noncompetitive binding site. In addition, several van der Waals interactions occur with the hydrophobic surface formed by Ile-141, Met-143, Phe-209, Val-211, and Leu-118. The benzyl ring moiety of equol would interact with the residues in the activation loop of the nonphosphorylated MEK1. Val-211, Leu-215, and Met-219 can form van der Waals interactions with the benzyl ring of the inhibitor. The hydroxyl groups at the 4’ position cannot form any hydrogen bonds with the activation loop in the model structure of MEK1. However, we cannot exclude the possibility that the binding of equol to MEK1 would induce a slight structural change of the activation loop to form a hydrogen bond with the hydroxyl groups at the 4’ position of equol. This is because the activation loop has an intrinsic flexibility as observed in many inactive kinase structures, in which their activation loops are disordered. The putative interactions between equol and the activation loop would lock MEK1 into a catalytically inactive formation by stabilizing the inactive conformation of the activation loop. The only structural difference between daidzein and equol is the additional carbonyl group at the 4 position (Fig. 1). When daidzein is superposed on equol docked to MEK1, the carbonyl group of daidzein would collide with the hydrophobic surface formed by Phe-209, Val-211, and Leu-118 (Fig. 9B). This steric collision would allow daidzein to bind to MEK1 much less effectively compared with equol, which would lead to the low inhibitory activity of daidzein. Further studies with x-ray crystallography to determine the inhibitor complex structure would elucidate the exact binding mode of equol to MEK1 and an explanation for its higher potency compared with daidzein.

In summary, equol is a metabolite of daidzein, which is more effective and less toxic at inhibiting TPA-induced cell transformation of JB6 P+ cells. This inhibition is associated with the suppression of MEK1 kinase activity, suggesting that MEK1 is a critical target for equol in mediating AP-1 activity and cell transformation. This represents the first report related to the molecular basis of the cancer-preventive action of equol, with our findings representing a significant step forward in our understanding of the mechanistic relationship between daidzein and equol and shedding new light on the molecular mechanisms of equol in chemoprevention.

Acknowledgment—We thank Andria Hansen for secretarial assistance.

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