



Equol induced apoptosis via cell cycle arrest in human breast cancer MDA-MB-453 but not MCF-7 cells

EUN JEONG CHOI and TAEHEE KIM

Plant Resources Research Institute, Duksung Women's University, 419 Ssangmun-dong, Tobong-ku, Seoul 132-714, Korea

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Abstract. To investigate the effects of equol on cell cycle distribution and apoptosis in human breast cancer cells, we first determined the antiproliferative effects of various concentrations of equol (1 nM to 100 μ M) on MCF-7 and MDA-MB-453 cells at 24, 48 or 72 h of exposure. Equol significantly inhibited proliferation in MDA-MB-453 cells in a dose- and time-dependent manner. In contrast, equol at low concentrations (<1 μ M) stimulated proliferation in MCF-7 cells, with increased expression of proliferating cell nuclear antigen (PCNA), and only inhibited proliferation at a high concentration (100 μ M). Similarly, equol treatment of MDA-MB-453 cells resulted in significant cell cycle arrest at the G₁/S transition and in the G₂/M phase, whereas it caused the slight cell cycle arrest of MCF-7 cells in the G₂/M phase after 72 h of treatment. Also, when cells were treated with 50 and 100 μ M equol for 72 h, the equol affected cell cycle regulatory proteins more significantly in MDA-MB-453 than in MCF-7 cells. During equol-induced apoptosis, equol increased the number of cells in the sub-G₀ phase and enhanced the level of p53. The expression of Bax and cytochrome c, downstream targets of p53, was markedly increased by treatment with higher concentrations of equol. Equol-induced cell cycle arrest and apoptosis apparently involves a p53-dependent pathway in different types of breast cancer cells.

Introduction

Several researchers have demonstrated associations between cell proliferation and the cell cycle in cancer (1,2), such that inhibitory effects on the proliferation of cancer cells can occur through the differential regulation of the cell cycle, leading to apoptosis or necrosis. A number of reports have also suggested that cell cycle control points, such as the G₁/S and G₂/M transitions, are potential targets for chemoprevention and cancer treatment in humans (3-5).

Correspondence to: Dr Eun Jeong Choi, Plant Resources Research Institute, Duksung Women's University, 419 Ssangmun-dong, Tobong-ku, Seoul 132-714, Korea
E-mail: ejchoi@duksung.ac.kr

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Recently, flavonoids have been proposed as potential chemotherapeutic agents owing to their biological activities, which include effects on cell cycle arrest and apoptosis induction (6-8).

Epidemiological studies have indicated that Asian populations consuming higher amounts of isoflavones have a lower incidence of cancer and cardiovascular disease than do Western populations, who consume lower amounts of isoflavones (9-13). Equol (7-hydroxy-3-(4'-hydroxyphenyl)-chroman) is a major metabolite of the isoflavone daidzein. Interestingly, some people are able to metabolize daidzein into equol (equol producers), and others are not (non-equol producers). Like other flavonoids, equol has antioxidant activity and the ability to act as a hydrogen/electron donor; it can thus scavenge free radicals (14-16). Equol also possesses estrogenic activity, with affinity for both estrogen receptors, ER α and ER β (17-19).

However, until recently the biological effects of equol have not been as well understood as those of daidzein. Only a few reported studies are available regarding the antiproliferative effects of equol and the mechanisms through which it promotes cell cycle arrest. In the present study, we investigated the antiproliferative effects of equol and explored the cell cycle control mechanism underlying these effects in breast cancer MCF-7 and MDA-MB-453 cells.

Materials and methods

Cell culture and equol treatment. Human breast cancer MCF-7 and MDA-MB-453 cells were purchased from the KCLB (Korean Cell Line Bank, Korea). Cells were routinely maintained in RPMI-1640 (Gibco), supplemented with 10% FBS and antibiotics (50 U/ml of penicillin and 50 μ g/ml streptomycin, Gibco) at 37°C in a humidified atmosphere containing 5% CO₂. In cell proliferation and cell cycle analysis experiments, cells were treated with either equol or vehicle alone for 24, 48 and 72 h. Daidzein and equol were purchased from Sigma and LC Laboratories (MA, USA), respectively, and dissolved in DMSO (final concentration 0.1% in medium).

Cell proliferation. Cell proliferation was determined using the MTT assay. At 24, 48 and 72 h, the cells exposed to equol were added to methylthiazolyl tetrazolium (MTT). Four hours later, DMSO was added to each well to dissolve the resulting formazan crystals, and then absorbance was recorded at 540 nm in a microplate reader (SpectraMax Plus; Molecular Devices).

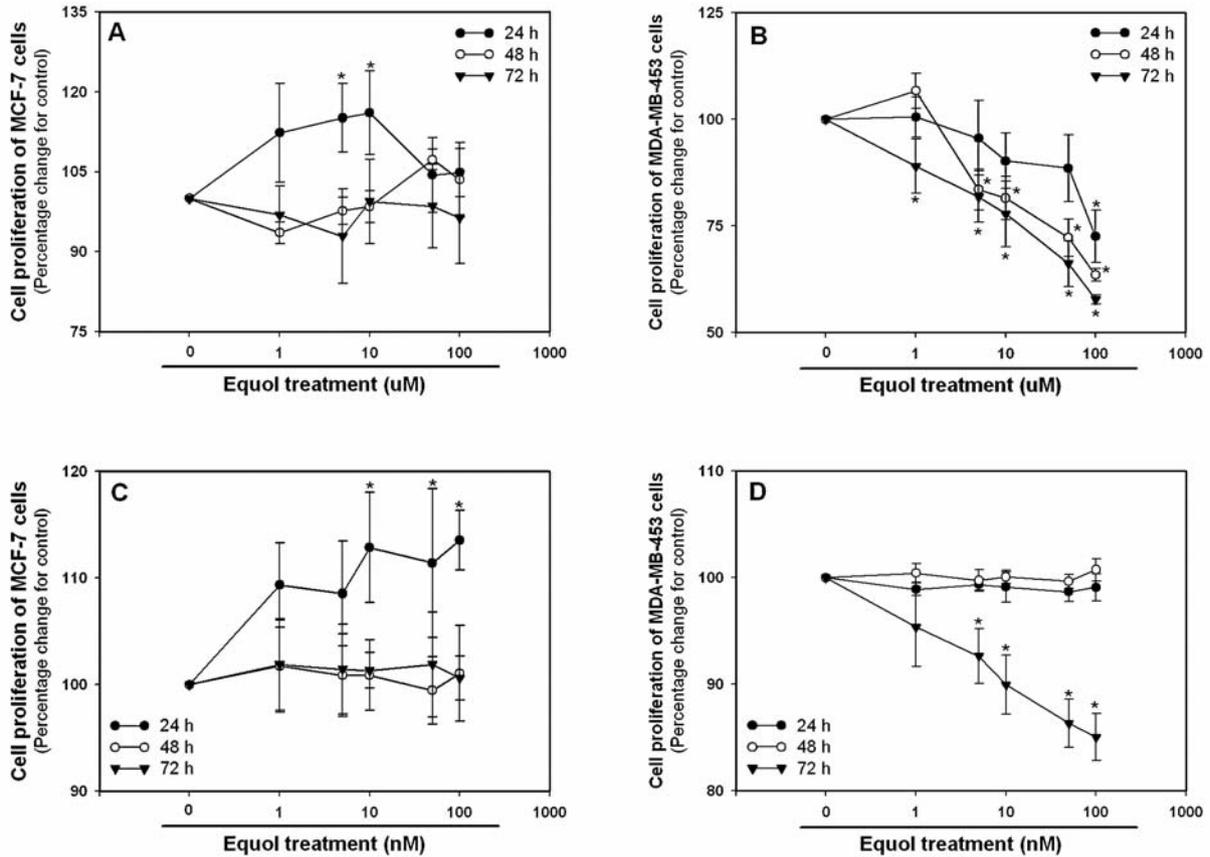


Figure 1. Effect of equol on cell proliferation in human breast cancer MCF-7 (A and C) and MDA-MB-453 (B and D) cells. Cells were exposed to equol at various concentrations (ranging from 1 nM to 100 μ M) and incubated for 24, 48 and 72 h. All data were expressed as the percentage change in comparison with the vehicle-only group, which was arbitrarily assigned 100% viability. * $P < 0.05$, significantly different from the vehicle-only group (0.1% DMSO in medium; i.e., an equol concentration of 0).

Cell cycle distribution. Cells were harvested, washed with cold PBS and processed for cell cycle analysis. Briefly, the cells were fixed in absolute ethanol and stored at -20°C for later analysis. The fixed cells were centrifuged at 1,000 rpm and washed twice with cold PBS. RNase A (20 $\mu\text{g}/\text{ml}$ final concentration) and propidium iodide staining solution (50 $\mu\text{g}/\text{ml}$ final concentration) was added to the cells and incubated for 30 min at 37°C in the dark. The cells were analyzed with a FACScalibur instrument (BD Biosciences, San Jose, CA) equipped with CellQuest 3.3 software. ModFit LT 3.1 trial cell cycle analysis software was used to determine the percentage of cells in the different phases of the cell cycle.

Immunoblotting. Protein expression was determined by Western blotting. Briefly, cells were lysed in RIPA buffer (1% NP-40, 150 mM NaCl, 0.05% DOC, 1% SDS, 50 mM Tris, pH 7.5) containing protease inhibitor for 1 h at 4°C . The supernatant was separated by centrifugation and protein concentration was determined by the Bradford Protein Assay Kit II (Bio-Rad Laboratories, CA, USA). Proteins (25 $\mu\text{g}/\text{well}$) denatured with sample buffer were separated by 10% SDS-polyacrylamide gel, then 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, then incubated with the primary

antibody overnight at 4°C . Primary antibodies and the house-keeping gene β -actin were purchased from Cell Signaling Technology 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. One specific protein band for PCNA was revealed by the Enhanced Chemiluminescence Kit (Amersham). The others were detected by the Opti-4CN Substrate Kit (Bio-Rad).

Statistical analyses. All data were expressed as a percentage compared to vehicle-treated control cells, which were arbitrarily assigned 100%. Data were analyzed by one-way analysis of variance followed by Dunnett's multiple comparison test (SigmaStat, Jandel, San Rafael, CA, USA). For all comparisons, differences were considered statistically significant at $p < 0.05$.

Results

Inhibition of cell proliferation by equol. The effects of equol on proliferation were assessed in human breast cancer MCF-7 and MDA-MB-453 cells, by MTT assay. At concentrations of 1-100 μM , equol significantly inhibited cell proliferation in a dose- and time-dependent manner in MDA-MB-453 cells,

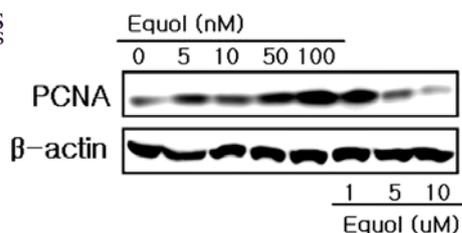


Figure 2. Effect of equol on PCNA expression in human breast cancer MCF-7 cells. Cells were exposed to equol at various concentrations (ranging from 5 nM to 10 μ M) and incubated for 24 h.

but not in MCF-7 cells (Fig. 1A). Significant anti-proliferative effects were observed at 24 h in MDA-MB-453 cells treated with 100 μ M equol. After more than 48 h of exposure, cell proliferation was significantly decreased at low equol concentrations ($p < 0.05$). Treatment with equol at a high concentration for 72 h decreased MDA-MB-453 cell proliferation by up to 57.7% compared to the controls. In contrast, cell proliferation was significantly increased in MCF-7 cells treated with 1 to <10 μ M equol for 24 h, although equol did not affect cell proliferation at 48 or 72 h (Fig. 1B).

To further evaluate the effect of equol on cell proliferation, MCF-7 and MDA-MB-453 cells were treated with very low

concentrations (1-100 nM) for 24, 48 and 72 h. In MCF-7 cells, equol at very low concentrations significantly increased cell proliferation at 72 h, although there was no change at 24 or 48 h. Specifically, exposure to 10, 50 and 100 nM equol decreased cell proliferation by 12.8, 11.4 and 13.5%, respectively, compared with the vehicle-only group ($p < 0.05$, Fig. 1C). In MDA-MB-453 cells, equol at very low concentrations showed a significant antiproliferative effect only at 72 h (Fig. 1D).

Promotion of cell proliferation by equol in MCF-7 cells. To confirm the MTT assay results regarding cell proliferation, MCF-7 cells were treated with equol at various concentrations (5 nM to 10 μ M) for 24 h (Fig. 2) and the expression of proliferating cell nuclear antigen (PCNA), a biomarker of cell proliferation, was determined. Increased PCNA expression was observed with equol treatments of 50 nM to 1 μ M. Very low PCNA expression occurred at 5 and 10 μ M equol.

Equol-induced cell cycle arrest. Exposure to 1-100 μ M equol for 72 h induced significant and dose-dependent cell cycle disruption in MDA-MB-453 cells, but not in MCF-7 cells (Fig. 3). Equol treatment resulted in a decreased number of MDA-MB-453 cells in the G_1/S and G_2/M phases. The G_1/S transition was significantly observed at 5 μ M, as the G_1 phase

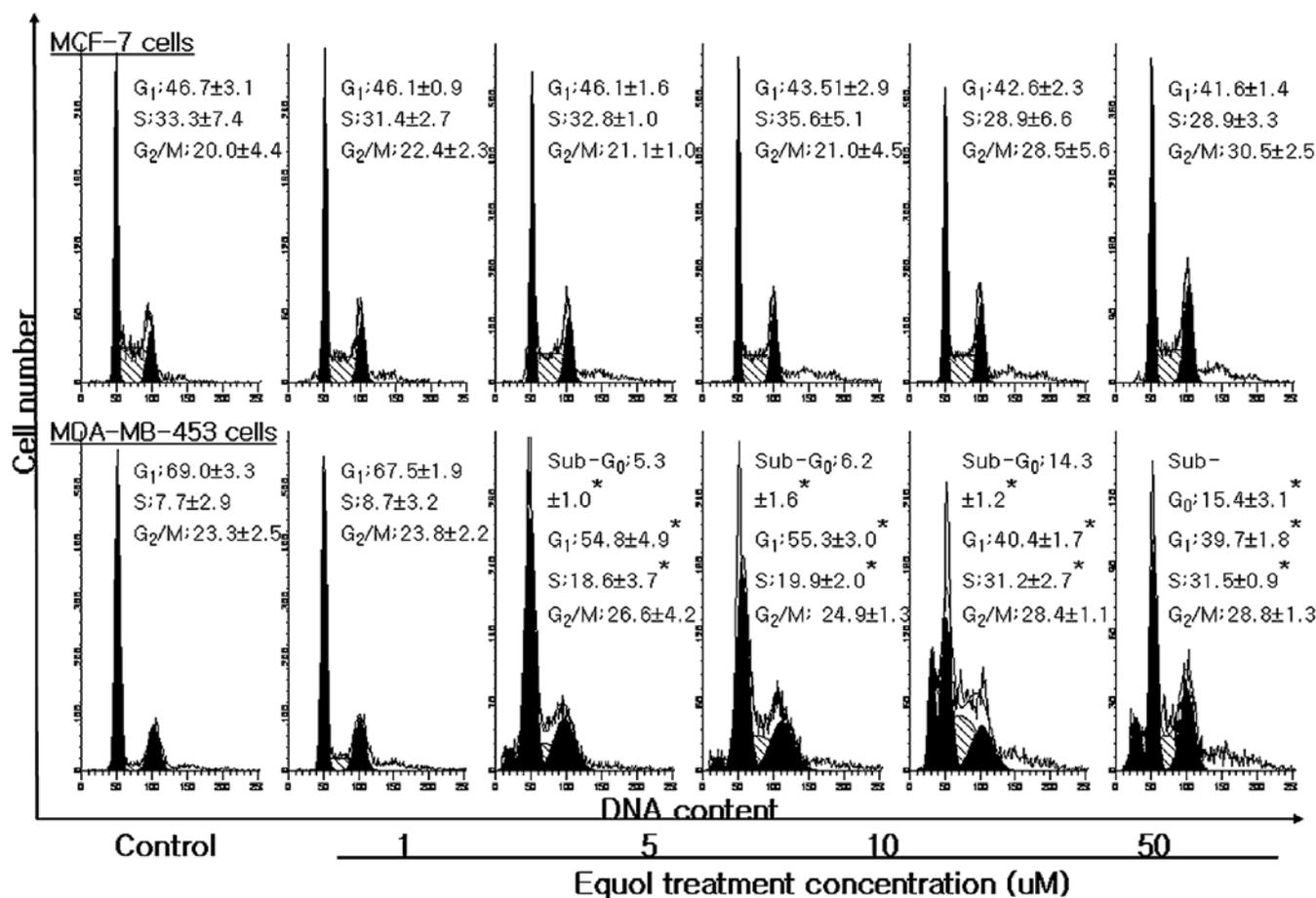


Figure 3. Effect of equol on cell cycle arrest in human breast cancer MCF-7 and MDA-MB-453 cells. Cells were exposed to equol at various concentrations (ranging from 1 to 100 μ M) and incubated for 72 h. * $P < 0.05$, significantly different from the vehicle-only group (0.1% DMSO in medium; i.e., an equol concentration of 0).

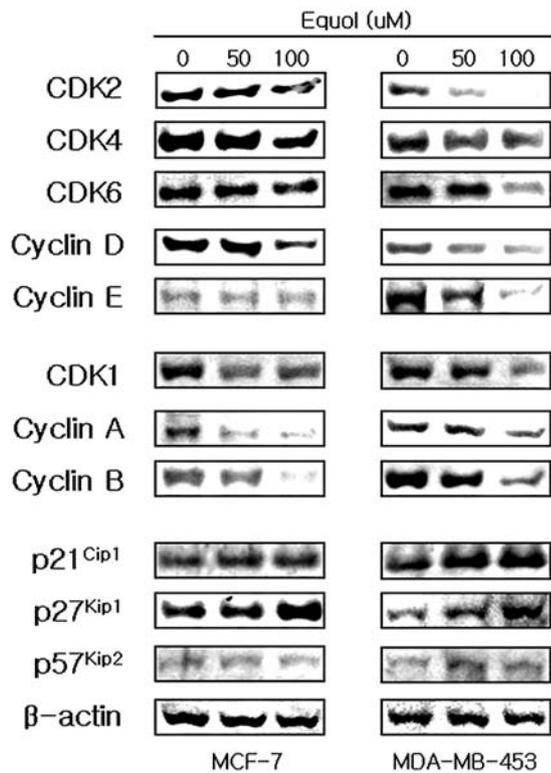


Figure 4. Effect of equol on cell cycle regulator protein expression in human breast cancer MCF-7 and MDA-MB-453 cells. Cells were exposed to equol at high concentrations (50 and 100 μM) and incubated for 72 h.

was accompanied by an increase in cell population in the S phase. After exposure to 5, 10, 50 and 100 μM equol, the proportion of MDA-MB-453 cells in the G₁ phase decreased from 69% to 54.8, 55.3, 40.4 and 39.7%, respectively, and the proportion of S-phase cells increased from 18.6% to 26.6, 19.2, 31.2 and 31.5%, respectively. At 50 and 100 μM equol, respectively, the G₂/M population was slightly increased by 21.8 and 23.6% compared with the controls. A significant increase in the number of MDA-MB-453 cells in the sub-G₀ phase was also observed after exposure to 5 μM equol. After treatment with higher equol concentrations (50 and 100 μM), small DNA fragments in the sub-G₀ phase increased from 0.9% to 14.3 and 15.4%, respectively.

In MCF-7 cells, significant cell cycle arrest in the G₂/M phase (30.5% of MCF-7 cells compared to 20.0% of the control cells, $p < 0.05$) occurred only after treatment with 100 μM equol for 72 h. Compared with the control population, the MCF-7 cell population in the G₂/M phase decreased by 42.5 and 52.5% at 50 and 100 μM equol, respectively.

Effects of equol on cell cycle-related protein expression. We examined the specific regulatory proteins responsible for equol-induced cell cycle arrest in cells treated with high concentrations of equol (50 and 100 μM) for 72 h (Fig. 4). In MCF-7 cells, equol treatment resulted in a slight reduction in the expression of cyclin D and CDK4 and no detectable change in cyclin E, CDK2, and CDK6 expression, compared to their expression in the control cells. In contrast, marked decreases occurred in the expression levels of CDK1 and cyclins A and

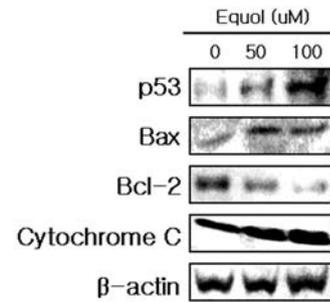


Figure 5. Effect of equol on apoptosis-related gene expression of human breast cancer MDA-MB-453 cells. Cells were exposed to equol at high concentrations (50 and 100 μM) and incubated for 72 h.

B - which combine with CDK1 to control the G₂/M phase - especially with 100 μM equol (Fig. 4B). At maximal treatment with 100 μM equol, cyclin B expression was especially decreased.

In MDA-MB-453 cells, 50 and 100 μM equol affected regulatory protein expression, markedly decreasing CDK2 and cyclin E expression in a dose-dependent manner. CDK6 expression was only decreased at 100 μM , whereas the expression of CDK4 and cyclin D was decreased regardless of equol concentration. Decreased CDK1 expression together with decreased expression of cyclins A and B was also observed, as in the MCF-7 cells. In addition, the expression of three inhibitors of cyclin-dependent kinase, p21^{Cip1}, p27^{Kip1} and p57^{Kip2}, increased dose-dependently in response to equol treatment in MDA-MB-453 cells (Fig. 4).

Equol-induced apoptosis. MDA-MB-453 cells were used to investigate equol-induced apoptosis because a strong sub-G₁ peak was present in these cells. When MDA-MB-453 cells were treated with high concentrations (50 and 100 μM) of equol for 72 h, p53 expression increased gradually in a dose-dependent manner. Additionally, the expression of downstream p53 targets such as Bax and cytochrome c increased markedly at both 50 and 100 μM equol (Fig. 5). Under the same conditions, Bcl-2 expression also decreased in a dose-dependent manner.

Discussion

Recently, equol has been considered an important player in the bioactive mechanism of isoflavones such as daidzein, although until now little information has been reported. In the present study, equol affected cell proliferation in different ways, depending on the type of human breast cancer cells used and the absence or presence of estrogen receptors.

Equol significantly inhibited cell proliferation in estrogen receptor-negative MDA-MB-453 cells in a dose- and time-dependent manner. This finding is consistent with previous reports indicating that daidzein may be an effective agent against cell growth in some cancer cell types (20,21). Thus, equol apparently has antiproliferative effects similar to its pro-drug, daidzein.

However, at very low concentrations, equol induced proliferation in MCF-7 cells, which have estrogen receptors. This result was supported by increased PCNA expression in

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 Cells treated with low concentrations of equol. PCNA in MCF-7 cells also increased with 50 nM to 1 μ M equol treatment for 24 h. PCNA is an essential replication factor, synthesized at the initial stages of the G₁ phase, and has a long half-life, accumulating in the nucleus until mitosis. PCNA is related to the initiation of cell cycle progression and is a useful marker of cell proliferation (22).

Previous studies have suggested that equol, acting as an estrogen-like bioactive molecule, might stimulate tumor cell growth, given that equol has stronger *in vitro* estrogenic properties than daidzein (23). Additionally, equol has been reported to show proliferative effects on MCF-7 cell growth *in vitro* (24,25), consistent with our results indicating that the exposure of MCF-7 cells to equol significantly stimulated cell proliferation.

To investigate the apoptosis pathway via cell cycle arrest induced by equol, we first analyzed cell cycle arrest in human breast cancer MCF-7 and MDA-MB-453 cells at various equol concentrations (1-100 μ M) for 72 h. In MCF-7 cells, cell cycle arrest was not induced at low concentrations of equol, but significant G₂/M phase arrest was induced at the maximal concentration (100 μ M). Additionally, in support of G₂/M phase arrest, there were marked decreases in the amount of CDK1 (Cdc2) protein, cyclin A and cyclin B, but levels of G₁ phase-related cell cycle regulator proteins, such as CDK2, CDK4 and cyclin D, were only slightly decreased by equol treatment. CDK1 is a catalytic subunit of the M-phase promoting factor, which is activated at the G₂/M transition and controls the onset of mitosis. Several investigators have shown that CDK1, in combination with cyclins A and B, is important in the G₂/M phase transition (26,27). In MCF-7 cells treated with equol, down-regulation of CDK1 may be a major cause of G₂/M phase arrest.

In contrast, profound differences were found in the cell cycle arrest of MDA-MB-453 cells in response to equol. These results suggest that equol can induce cell cycle arrest through an ER-independent pathway, rather than an ER-dependent one, because MDA-MB-231 cells are ER α -negative, as mentioned above. In the present study, equol caused G₁/S transition, together with S-phase progression, in MDA-MB-453 cells, and DNA accumulation in the sub G₀/G₁ phase was also observed at low concentrations of equol. It has been reported that some flavonoids have the ability to arrest cell growth at more than one stage of the cell cycle (28-31).

Equol affected cell cycle regulatory proteins more significantly in MDA-MB-453 cells than in MCF-7 cells. Cell cycle progression is regulated by the activity of cyclin-dependent kinases (CDKs). At the G₁/S transition of the cell cycle, cyclins D, E and A associate with CDKs to promote cell cycle progression. The activation of CDK2/cyclin E, which is necessary for DNA replication, is particularly important at the G₁/S transition. CDKs are also regulated in part by the cyclin-dependent kinase inhibitors (CKIs), which bind to cyclin/CDK complexes to inhibit CDK activity. There are two CKI families: one contains p21^{Cip1}, p27^{Kip1} and p57^{Kip2}; and the other the INK4 family, including p16^{INK4a}, p15^{INK4b}, p18^{INK4c} and p19^{INK4d} (32). CDK2 is the major kinase that allows progression through the G₁/S phase and subsequent replication events.

Equol induced a decrease in the expression of CDK2, CDK4, cyclin E and cyclin D, together with a slight decrease in the expression of G₂/M-related regulators such as CDK1, cyclin A and cyclin B. At the maximal equol concentration, the level of CDK2 was almost zero. CDK2 appears to play a key role in equol-induced G₁/S phase cell cycle arrest.

Additionally, equol induced a dose-dependent increase in p21^{Cip1} and p27^{Kip1} expression in MDA-MB-453 cells. The induction of p27 has been shown to be closely related to the G₁ transition in cell cycle arrest. It has been reported that p27^{Kip1}, of the Cip/Kin family of CKIs, is regulated by CDK2/cyclin E to prevent premature entry into the S phase (33,34).

To further examine equol-induced apoptosis, apoptosis-related proteins were investigated in MDA-MB-453 cells, which demonstrated significant cell cycle arrest and DNA fragmentation in the sub-G₀. In the present study, equol dose-dependently increased p53 expression in MDA-MB-453 cells. 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 (35). The activation of p53 in response to DNA damage leads to cell cycle arrest and the inhibition of cell proliferation (36,37). Members of the Bcl-2 family of proteins, located downstream of p53, are critical regulators of the apoptotic pathway (38,39). These proteins consist of the major antiapoptotic family members Bcl-x(L) and Bcl-2, and the major pro-apoptotic proteins Bax and Bak. After exposure to equol (50 and 100 μ M), Bax expression increased along with a decrease in Bcl-2 expression. Bax controls mitochondrial permeability and cytochrome c expression, and the release of cytochrome c from mitochondria to the cytoplasm is a key step in the initiation of apoptosis. Our results showed that the activity of cytochrome c was significantly increased in MDA-MB-453 cells following treatment with equol.

In conclusion, our results indicate that equol significantly inhibited cell proliferation and induced cell cycle arrest and apoptosis in ER-negative MDA-MB-453 cells. Considering that equol stimulated or inhibited proliferation in MCF-7 cells depending on the concentration, equol had stronger effects in estrogen-independent pathways. Additionally, this study suggests, for the first time, the mechanisms by which equol induces cell cycle arrest and apoptosis. We propose the existence of multiple pathways, via which equol treatment leads to cell cycle arrest at the G₁/S transition as a result of up-regulated p27^{Kip1} and down-regulated CDK2, in MDA-MB-453 cells. Equol also produces G₂/M arrest via down-regulated CDK1 in both MCF-7 and MDA-MB-453 cells. Thus, equol operates by different pathways in different subtypes of breast cancer. Moreover, the p53 pathway and up-regulation of cytochrome c expression may be involved in the anti-cancer pathway of equol-induced apoptotic cell death in MDA-MB-453 cells.

Acknowledgements

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