Anti-/pro-apoptotic effects of hesperetin against 7,12-dimetylbenz(a)anthracene-induced alteration in animals

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Abstract. This study investigated the anticancer effects of hesperetin on 7,12-dimethylbenz(a)anthracene (DMBA)-treated animals and explored its anticancer mechanism. The experiment consisted of two parts. First, Sprague-Dawley rats were given hesperetin daily at a dose of 50 mg/kg for 8 weeks after a single dose of DMBA (100 mg/kg). As controls, rats were divided into vehicle alone and DMBA alone groups. Secondly, ICR mice were given hesperetin daily at a dose of 10 and 50 mg/kg BW/day for 7 weeks before a single dose of DMBA (34 mg/kg/week). In rats with DMBA-induced mammary gland tumors, hesperetin pretreatment significantly reduced the tumor burden and PCNA overexpression. The administration of hesperetin significantly inhibited mammary gland carcinoma from developing by restoring the decreased Bcl-2 and increased Bax expression. By contrast, in the livers of mice treated with DMBA, obvious DNA fragmentation was observed. Moreover, apoptosis-related gene expression in the livers of the mice differed from that in mammary gland carcinomas in rats. These changes were restored in mice treated with hesperetin, indicating the inhibition of apoptosis. Based on these results, hesperetin may act not only as a proapoptotic agent, but also as an antiapoptotic agent, depending on the circumstance.

Introduction

Free radicals and/or ROS are thought to be involved in a number of pathophysiological conditions. The most deleterious effects of ROS, which include lipid peroxidation, protein oxidation, and oxidative DNA damage, may have an etiological or prognostic role in cancer (1). Recently, the antiproliferative and proapoptotic effects of antioxidants from plants have been explored in an attempt to create new chemopreventive and chemotherapeutic agents. Multiple studies have demonstrated that flavonoids can suppress cancer through their antioxidative, antiproliferative, and proapoptotic effects (2,3). The ability of flavonoids to promote apoptosis makes them very attractive candidates in the development of anticancer drugs.

Among these flavonoids, hesperetin (3',5,7-trihydroxy-4-methoxyflavanone), which is occurs as hesperidin (its glycoside form) in nature, and is found in fruit sources, including various citrus species (4,5). Although few *in vitro* studies have compared hesperetin with hesperidin, we previously found that hesperetin inhibits cellular proliferation, the induction of a cell cycle arrest at G_1, and apoptosis in human breast MCF-7 cancer cells (6). Based on these data, hesperetin may be a good candidate for an anticancer drug; however, no *in vivo* data are available concerning its possible anticancer effects.

Therefore, we investigated the anticancer effects of hesperetin in an animal model using a chemical carcinogen. Moreover, the mechanism of the anticancer effects of hesperetin via antioxidation and the induction of apoptosis was explored.

First, the proapoptotic effect of hesperetin 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 (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 (7).

Second, the effects of hesperetin in modulating the cancerous response to oxidative stress was investigated in the livers of mice exposed to DMBA for 1 week after hesperetin pretreatment. 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 (8,9). In addition, DMBA induces substantial oxidative damage as result in the formation of ROS such as peroxides, hydroxyl, and superoxide anion radicals in organs such as the liver and mammary glands (10-12). This study was designed to evaluate the potential benefit of

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hesperetin as a chemopreventive and/or chemotherapeutic agent for human cancer cells.

Materials and methods

Hesperetin treatment 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 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. Hesperetin (Sigma) was dissolved in water and administered orally to rats at a dose of 50 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 hesperetin daily at a dose of 10 and 50 mg/kg BW for 7 weeks before a single dose of DMBA (34 mg/kg/week). After hesperetin administration was followed for 1 week continuously. The control groups were the same as above and each group 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. The hesperetin treatment dosage was determined based on a previous study, where serum glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT), biomarkers of hepatic function, were not affected in mice treated with a hesperatin dose of 50 mg/kg BW for 12 weeks.

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 experiment, mice were rapidly anesthetized using ether at 6 h after final administration of hesperetin. Their livers and mammary gland tumors were isolated, blotted, weighed, frozen in liquid nitrogen and stored at -70˚C until assayed. Mammary gland tumor volume was measured using calipers and calculated as following formula: $V=4/3\pi r^3$.

Immunoblotting assay. Samples were homogenizing with in RIPA buffer (1% NP-40, 150 mM NaCl, 0.05% DOC, 1% SDS, 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 Bcl-2, Bax, cleaved caspase-9 (37 kDa), cleaved caspase-3 (19 kDa), cleaved PARP (85 kDa) and ß-actin were purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and used to probe the separate membranes. The next day, the immunoreaction was continued with 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).

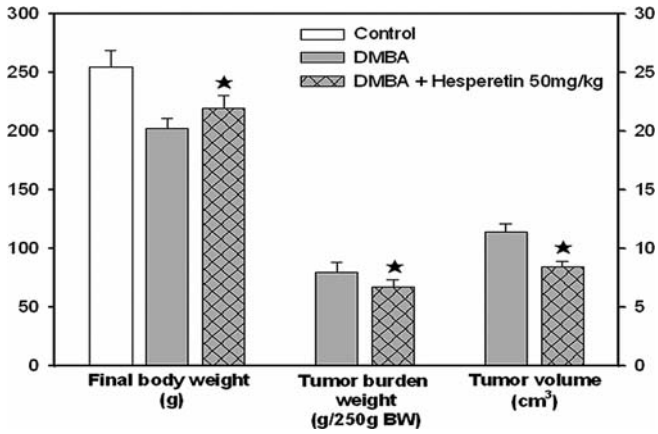

Figure 1. Effects of hesperetin on body weight, tumor weight and volume in rats mammary gland tumors. Sprague-Dawley rats were given hesperetin daily at a dose of 50 mg/kg for 8 weeks after a single dose of DMBA (100 mg/kg). As controls, rats were divided into vehicle alone and DMBA alone groups. Values are presented as mean ± SD (n=8). *P<0.05, significantly different from the respective control group (DMBA-treated or untreated).

DNA fragmentation assay. Samples were homogenized in lysis buffer (20 mM EDTA, 0.5% CHAPS, 100 mM Tris, pH 8.0) and were 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 follow. 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.

ROS generation assay. Samples were homogenized in ice cold isolation buffer (320 mM sucrose, 1 mM EGTA, 10 mM Tris, pH 7.4) and centrifuged for 5 min at 1,000 x g. Then, the supernatant was transferred and centrifuged for 5 min at 7,800 x g. The pellet was resuspended in isolation buffer containing 19% Percoll and centrifuged for 10 min at 11,200 x g. The pellet form samples were washed again with cold PBS and processed for ROS generation analysis. Reactive oxygen species (ROS) were determined by using fluorescent probe H_2DCF-DA (Molecular Probes, Eugene, OR). H_2DCF-DA is enzymatically hydrolyzed by intracellular esterases to form non-fluorescent H_2DCF, which is then rapidly oxidized to form highly fluorescent DCF in the presence of ROS. Briefly, the cells were fixed in absolute ethanol and stored at -20˚C for later analysis. Cells were washed again in cold PBS and incubated with 1 μM 2',7'-dichlorofluorescein diacetate (DCFH-DA, Sigma) at 37˚C for 15 min in the dark. The cells were analyzed a FACSCalibur instrument (BD Biosciences, San Jose, CA). The DCF oxidation was determined using the median fluorescence intensity on the FL1 detector.

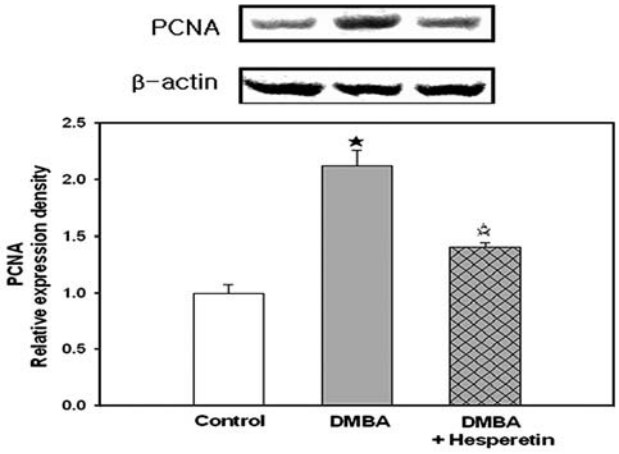

Figure 2. Effects of hesperetin on PCNA expression in rats mammary gland tumors. Sprague-Dawley rats were given hesperetin daily at a dose of 50 mg/kg for 8 weeks after a single dose of DMBA (100 mg/kg). As controls, rats were divided into vehicle alone and DMBA alone groups. Values are presented as mean ± SD (n=8). ★P<0.05, significantly different from the control group. ☆P<0.05, significantly different from the DMBA-treated group.

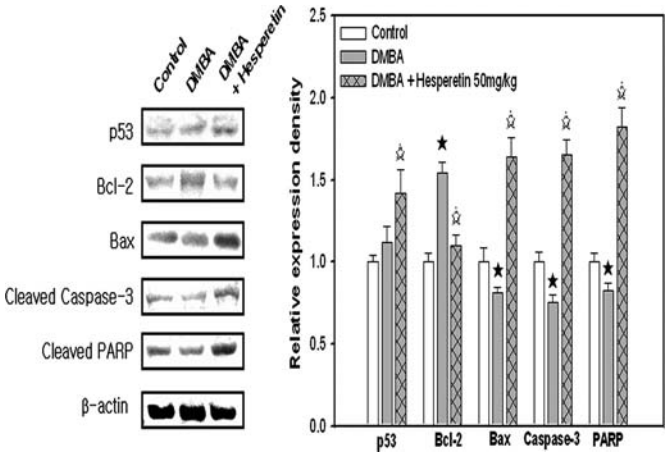

Figure 3. Effect of hesperetin on p53, Bcl-2, Bax, cleaved caspase-3 and PARP expressions in rats mammary gland tumors. Sprague-Dawley rats were given hesperetin daily at a dose of 50 mg/kg for 8 weeks after a single dose of DMBA (100 mg/kg). As controls, rats were divided into vehicle alone and DMBA alone groups. Values are presented as mean ± SD (n=8). ★P<0.05, significantly different from the control group. ☆P<0.05, significantly different from the DMBA-treated group.

Statistical analyses. Data were analyzed by unpaired Student's t-test or 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

Effects of hesperetin on body weight and tumor weight in rats with DMBA-induced mammary gland tumors. A significant decrease in body weight was observed in the DMBA-treated group compared with the control group (P<0.05). The average weight of the induced mammary gland tumors was 7.98 g/250 g BW (Fig. 1). Hesperetin significantly reduced the tumor burden by 19.6% as compared to rats treated with DMBA only (P<0.05). Tumor volume of hesperatin-treated rats (1.14 cm^3, P<0.05) was also significantly reduced as compared to the DMBA group (0.84 cm^3).

Effects of hesperetin on the expression of PCNA in rats with DMBA-induced mammary gland tumors. PCNA expression in the mammary gland tumors of rats treated with DMBA was significantly increased by 2.1 times compared with control rats (Fig. 2, P<0.05). The level of overexpression was reduced by ~50% in the hesperetin-pretreated group.

Pro-apoptotic effects of hesperetin on DMBA-induced mammary gland tumor cells. Hesperetin treatment significantly increased p53 expression by 21.1%, as compared to the DMBA group. There was no statistically significant difference in p53 expression between the DMBA group and controls. DMBA increased Bcl-2 expression in the mammary gland tumors of mice treated with DMBA by 54.1% compared with the control group (Fig. 3, P<0.05), whereas hesperetin treatment restored Bcl-2 expression to near-normal levels. Also, Bax expression was significantly decreased by 18.8% in mice treated with DMBA compared with the control group. Similarly, cleaved caspase-3 and PARP expression was decreased by 24.8 and 17.6%, respectively, compared with the control values. Bax, cleaved caspase-3, and PARP expression by hesperetin was elevated approximately 2-fold compared with DMBA groups.

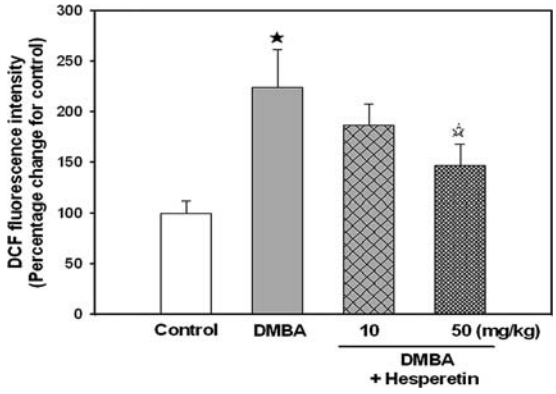

Figure 4. Effect of hesperetin on ROS generation in liver of mice treated with DMBA. ICE mice were given hesperetin daily at a dose of 50 mg/kg BW/day for 3 weeks after a single dose of DMBA (34 mg/kg). As controls, rats were divided into vehicle alone and DMBA alone groups. Values are presented as mean ± SD (n=6). All data are reported as the percentage change in comparison with the vehicle-only group, which were arbitrarily assigned 100% viability.

Effects of hesperetin on body weight and food intake in mice treated with DMBA. No statistically significant differences in body weight were observed between the DMBA group versus controls at the end of the experimental periods. Oral pre-administration of hesperetin to mice did not affect body weight or food intake (data not shown).

Effects of hesperetin on the ROS generation in mice treated with DMBA. ROS levels in the livers of mice exposed to

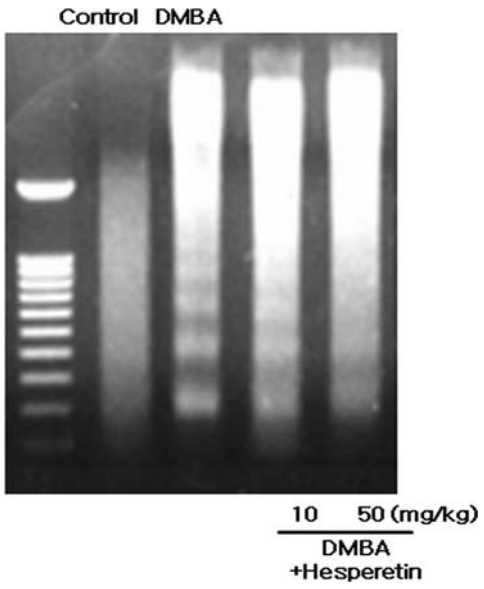

Figure 5. Effect of hesperetin on DMBA-induced DNA fragmentation in liver of mice treated with DMBA. ICE mice were given hesperetin daily at a dose of 50 mg/kg BW/day for 3 weeks after a single dose of DMBA (34 mg/kg). As controls, rats were divided into vehicle alone and DMBA alone groups. Values are presented as mean ± SD (n=6).

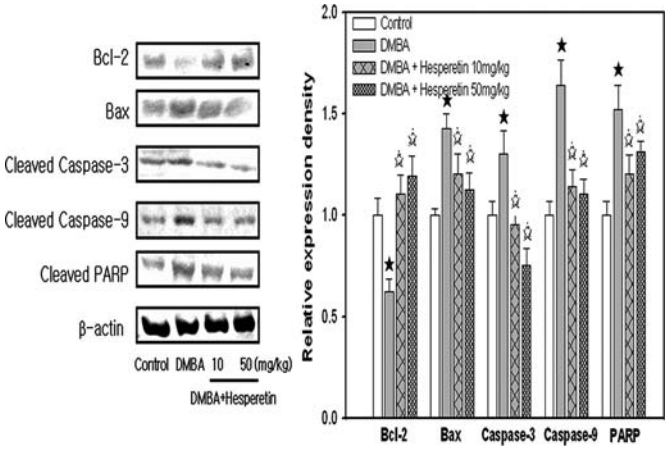

Figure 6. Effect of hesperetin on Bcl-2, Bax, cleaved caspase-3 and PARP expressions in liver of mice treated with DMBA. ICE mice were given hesperetin daily at a dose of 50 mg/kg BW/day for 3 weeks after a single dose of DMBA (34 mg/kg). As controls, rats were divided into vehicle alone and DMBA alone groups. Values are presented as mean ± SD (n=6). ★P<0.05, significantly different from the control group. ☆P<0.05, significantly different from the DMBA-treated group.

DMBA were significantly increased: 2.2 times that of controls (Fig. 4, P<0.05). In mice treated with the higher dose of hesperatin, however, this induction was reduced by ~34.5%.

Anti-apoptotic effects of hesperetin in mice treated with DMBA. DNA fragmentation was observed in the hepatic cells of mice exposed to DMBA (Fig. 5). Hesperetin pretreatment prevented DNA fragmentation efficiently, but not completely, in a dose-dependent manner. In the liver of mice exposed to DMBA, we observed a 37.5% decrease in Bcl-2 expression and a 42.5% increase in Bax expression compared with the control group (Fig. 6). In addition, cleaved caspase-3 and PARP expression was increased by 30.2 and 52.1% in the DMBA-treated group, respectively, compared with the control group. These changes were reversed by pretreatment with hesperetin; Bcl-2 expression was increased by 76.3 and 90.8%, as compared to the DMBA group after hesperetin pretreatment doses of 10 and 50 mg/kg BW, respectively. Hesperetin significantly decreased Bax, cleaved caspase-3, caspase-9, and PARP expression at the lower dose (10 mg/kg BW), regardless of exposure time (P<0.05).

Discussion

We investigated the mechanism through which hesperatin mediates its anticancer effects in the animal model. Using SD rats with mammary gland tumors induced by DMBA, tumor formation was decreased in the hesperetin-treated group in comparison with rats that received only DMBA. Cellular proliferation is an integral part of carcinogenesis, with important roles in several steps of the carcinogenic process, including initiation, promotion, and progression (13,14). 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 tumor of DMBA-treated rats. PCNA expression has also been used to measure the proliferation rate of tumors. PCNA expression was significantly increased in mammary gland tumors induced by DMBA compared with that in control rats. This is consistent with previous reports that showed PCNA overexpression in a wide range of human tumors (15,16), as well as in DMBA-induced mammary gland tumors (17,18). Hesperetin treatment significantly reduced PCNA overexpression by >50%. These data suggest that hesperetin decreases cell proliferation in mammary gland tumors through the reduction of PCNA expression.

We previously reported that hesperetin induced G1-phase cell cycle arrest *in vitro* through the regulation of CDK4 and $p21^{Cip1}$, suggesting that involvement of the apoptotic process is one possible mechanism through which hesperetin mediates its anticancer effects. In the present study, hesperetin treatment significantly increased the expression of p53, an upstream target of $p21^{Cip1}$, consistent with our previous data. The tumor suppressor gene p53 is regarded as a key factor in the balance between cell survival and cell death, via regulation of both the G_1 and G_2/M portions of the cell cycle (19). Activation of p53 in response to DNA damage would lead to cell cycle arrest and inhibition of cell proliferation, thereby preventing tumor formation (20,21).

Bcl-2 family proteins are critical regulators of the apoptotic pathway and control both mitochondrial permeability and cytochrome c expression (22,23). 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. In the DMBA-treated group, Bcl-2 expression was increased, whereas Bax expression was decreased. It has been reported that Bcl-2 overexpression inhibits the transcriptional activation of Bax (24). Overexpression of antiapoptotic proteins such as Bcl-2 may

contribute to tumor formation in DMBA-treated SD rats. Hesperetin treatment significantly induced apoptosis in the mammary gland tumors by increasing proapoptotic Bax expression, with a subsequent increase in the expression of cleaved caspase-3. 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 (25,26). Also, PARP is another characteristic hallmark of apoptosis, and PARP overexpression was also induced by hesperetin.

Flavonoids act as general cell growth inhibitors and apoptosis inducers, which makes them prime candidates in the development of new anticancer compounds (27-29). Also, several studies have shown that flavonoids are protective against DMBA-induced rat mammary tumorigenesis (30,31). Thus, the proapoptotic capacity of hesperetin in mammary gland tumors may be valuable in the development of new anticancer drugs.

In our second experimental model, we assessed the induction of oxidative liver damage in mice by DMBA because the production of ROS in hepatocytes is significantly elevated by DMBA. This is consistent with DMBA having a dose- and time-dependent effect on the production of H_2O_2 in rat liver microsomes (32). The formation of ROS during DMBA metabolism induces oxidative damage, the first step involved in mutagenesis, carcinogenesis and ageing. It is well established that ROS-mediated DNA damage is associated with carcinogenesis (33,34). Hesperetin pretreatment inhibited the production of ROS in a dose-dependent manner.

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 (35). The induction of DNA fragmentation by DMBA was reduced by hesperetin in a dose-dependent fashion. 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 (12); similarly, apoptosis was detected 24 and 36 h after DMBA treatment (80 mg/kg) in the adrenal cortexes of the mice (36).

Several recent reports have suggested that antioxidants play a major role in the development of cancer. Several mechanisms for the chemopreventive effects of antioxidants have been proposed, including the suppression of ROS generation and the activation of an antioxidant defense system during both the initiation and promotion of cancer (37-40). It has been reported that increased levels of antioxidants decrease ROS levels and inhibit apoptosis (41). We found that hesperetin pretreatment restored the expression of Bcl-2, Bax, cleaved caspase-3, caspase-9, and PARP to their normal levels, regardless of the dose. It has been shown that apoptosis in the adrenal cortex preceded necrosis after treatment of rats with DMBA (42). That is, in pathophysiological condition apoptosis can lead to induction of inflammation because of the onset of secondary necrosis, although apoptosis can occur in a physiological condition without inflammation (43,44). Previous studies report that caspase-9 is activated by reactive oxygen species (45). In the present study, hesperetin pretreatment remarkably inhibited the activation of caspase-9 by DMBA. This hespertin-mediated modulation of apoptosis-related gene expression in mice with DMBA-induced apoptosis may be the result of a protective mechanism in the liver.

In summary, our data support previous *in vitro* results that suggest the usefulness of hesperetin as an anticancer agent (6,46). In our *in vivo* study, hesperetin induced the apoptosis in mammary gland tumors. In addition, hesperetin showed a hepatic protective effect via the reduction of apoptosis in mice with oxidative liver damage. Thus, hesperetin may act not only as a pro-apoptotic agent, but also as an anti-apoptotic agent, depending on the circumstance. These 'two-faced' characteristics of hesperetin may be useful in the development of chemopreventive and/or chemotherapeutic agents.

Acknowledgements

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