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Exposure of breast cancer cells to a subcytotoxic dose of apigenin causes growth inhibition, oxidative stress, and hypophosphorylation of Akt

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ABSTRACT

Epidemiological studies show that fruit- and vegetable-rich diets are associated with a reduced risk of developing certain forms of cancer, including breast cancer. In this study we demonstrate that a subcytotoxic concentration of apigenin, which is a flavone found at high concentrations in parsley, onions, grapefruit, oranges, and chamomile tea, inhibited DNA synthesis in a panel of human breast cancer cell lines (MDA-MB-231, MBA-MB-468, MCF-7, SK-BR-3). Decreased proliferation of MDA-MB-468 cells in the presence of apigenin was associated with G_2/M phase cell cycle arrest and the production of reactive oxygen species. Apigenin-treated MDA-MB-468 cells also showed reduced phosphorylation of Akt (protein kinase B), which is an essential effector serine/threonine kinase in the phosphatidylinositide 3-kinase pathway that promotes tumor growth and progression. However, exposure to the antioxidant reduced glutathione failed to reverse apigenin-mediated inhibition of Akt phosphorylation and cell proliferation, indicating that these effects were not due to oxidative stress. Taken together, these findings suggest that low-dose apigenin has the potential to slow or prevent breast cancer progression.

1. Introduction

Although recent years have seen tremendous progress in the detection and treatment of breast cancer (Howard and Bland, 2012), this disease remains the most common malignancy and the predominant cause of cancer-related death in women (Are et al., 2013). Treatment recommendations are currently based on whether or not a breast tumor expresses human epidermal growth factor receptor 2 (HER2), estrogen receptor and/or progesterone receptor; however, molecular profiling has recently revealed that breast cancer is a heterogeneous and phenotypically diverse malignancy (Cyr and Margenthaler, 2014). Treatment options consist of surgery with adjunct radiotherapy, chemotherapy, hormone therapy, and/or targeted biologic therapy (Howard and Bland, 2012). Breast cancers that express estrogen receptor are typically treated with drugs that inhibit receptor signaling or estrogen production whereas HER2-overexpressing tumors are treated with the HER2-specific monoclonal antibody trastuzumab. Breast cancers that fail to express estrogen receptor, progesterone receptor, and HER2 (triple-negative) initially respond well to chemotherapy but have a poor prognosis upon recurrence due to their lack of sensitivity to hormone therapy or HER2-targeted treatment (Foulkes et al., 2010). Unfortunately, breast cancers frequently become resistant to chemotherapy due to the emergence of multidrug-resistant variants (Bush and Li, 2002). In addition, current systemic breast cancer treatments also have a number of adverse side effects that include cardiac toxicity (Ades et al., 2014) and reduced quality of life due to musculoskeletal and menopausal symptoms (Henry, 2014). More effective and better tolerated adjunct treatments for breast cancer are therefore of considerable interest to both patients and clinicians.

Epidemiological studies indicate that a diet rich in fruits and vegetables is associated with a lower incidence of many types of cancer (Riboli and Norat, 2003), presumably because of the consumption of a multitude of bioactive plant compounds known as phytochemicals, many of which have potential additive or synergistic cancer-fighting activities (González-Vallinas et al., 2013; Liu, 2004; Weng and Yen, 2012). Apigenin (5,7,4′-trihydroxyflavone) is a low molecular weight polyphenolic flavone that is present in parsley, celery, onions, grapefruit, and oranges; however, apigenin is most abundant in chamomile tea, which contains 0.8–1.2% apigenin by weight/volume (Shukla and Gupta, 2010). Recent studies show that systemic administration of apigenin to Sprague–Dawley rats by intraperitoneal injection protects against the development of mammary tumors induced by treatment with 7,12-dimethylbenz(a)anthracene (Mafuvadze et al., 2011). Apigenin also preferentially induces apoptosis in HER2-overexpressing breast cancer cells by a mechanism that involves inhibition of the serine/

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threonine kinase Akt (protein kinase B) (Way et al., 2004), as well as inhibition of STAT3 and NFκB signaling pathways (Seo et al., 2012). Although the effect of apigenin on the in vivo growth of HER2-overexpressing breast cancer cells has not yet been tested, subcutaneous injections of apigenin impair the growth of MDA-MB-231 breast cancer cells in nude mice by proteasome inhibition and apoptosis induction (D. Chen et al., 2007). Importantly, apigenin does not have any adverse effects on cultures of normal human cells or exhibit any apparent toxicity in mice (D. Chen et al., 2007; Gupta et al., 2001).

Previous studies have shown that high concentrations of apigenin induce apoptosis in breast cancer cells (Choi and Kim, 2009; D. Chen et al., 2007; Seo et al., 2012; Way et al., 2004); however, there is less information regarding the impact of treatment with lower doses of apigenin on breast cancer cell growth. In this study, we determined that a subcytotoxic concentration of apigenin, which could possibly be achieved by oral dosing, inhibited the in vitro growth of human breast cancer cell lines.

2. Materials and methods

2.1. Cell lines

MDA-MB-231 human breast carcinoma cells were kindly provided by Dr. S. Dover (Memorial University of Newfoundland, St. John's, NL). MDA-MB-468, MCF-7, and SK-BR-3 human breast carcinoma cells were generous gifts from Drs. P. Lee, K. Goralski, and G. Dellaire, respectively (Dalhousie University, Halifax, NS). All breast cancer cell lines were authenticated by short tandem repeat profiling performed by ATCC (Manassas, VA). Cell cultures were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich Canada, Oakville, ON) supplemented with 10% heat-inactivated (56 °C for 30 min) fetal bovine serum, 5 mM HEPES buffer (7.4 pH), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Invitrogen Canada, Oakville, ON) at 37 °C in a humidified 10% CO_2 atmosphere.

2.2. Reagents

Apigenin, dimethyl sulfoxide (DMSO) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), reduced glutathione (GSH), and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich Canada. Apigenin was prepared in DMSO at stock concentrations of 20 mM and stored at −20 °C. Oregon Green 488 carboxylic acid diacetate, 2′,7′-dicholorofluorescin 3′6′-diacetate (DCF-DA), TrypLE, 0.25% trypsin-EDTA, and propidium iodide (PI) were all purchased from Invitrogen Canada. Acrylamide/bis-acrylamide (29:1, 30% solution), ammonium persulfate (APS), ethylene glycol tetraacetic acid (EGTA), sodium dodecyl sulfate (SDS), paraformaldehyde (PFA), tetramethylethylenediamine (TEMED), Tris base, and Tween-20 were purchased from Bio-Shop Canada Inc. (Burlington, ON). Rabbit anti-human-Akt and rabbit anti-human phospho-Akt (Ser 473) antibodies (Ab) were from Cell Signaling Technology (Danvers, MA). Horse radish peroxidase (HRP)-labeled bovine anti-rabbit IgG Ab was from Santa Cruz Technologies (Santa Cruz, CA).

2.3. Cell viability assay

An MTT assay, which measures succinate dehydrogenase activity, was used to determine breast cancer cell viability. Cells were seeded in quadruplicate in 96-well flat bottom plates at 1.2×10^4 cells/well and cultured in the absence or presence of apigenin for 24 h. Two hours before the end of the treatment period, 20 μl of MTT solution (5 mg/ml in PBS) was added to each well. Cells were then pelleted by centrifugation, supernatant was discarded, and 100 μl DMSO was added to each well to dissolve the formazan crystals. Absorbance was read at 570 nm using an Expert 96 microplate reader (Biochrom ASYS, Cambridge, UK). Percent viability of apigenin-treated cells was normalized to the medium control (100% viable cells), and calculated using the formula ([E / C] × 100), where E and C represent the absorbance readings of experimental and control samples, respectively.

2.4. DNA synthesis assay

Breast cancer cell synthesis of DNA was measured by tritiated thymidine ([^{3}H]TdR; MP Biomedicals, Santa Ana, CA) incorporation. Cells were seeded in quadruplicate in 96-well flat bottom plates at 1.2×10^4 cells/well or 6×10^3 cells/well for 24 h and 72 h culture, respectively, in the absence or presence of apigenin. Cultures were pulsed with 0.2 μCi [^{3}H]TdR for the last 6 h (MDA-MB-468, MDA-MB-231, MCF-7) or 18 h (SK-BR-3) of culture. To facilitate cell lysis, cells were frozen and thawed twice before harvesting onto fiberglass filter mats with a Titerek Cell Harvester (Skatron Instruments, Sterling VA). [^{3}H]TdR incorporation into newly synthesized DNA was measured by liquid scintillation counting using a Beckman LS6000IC liquid scintillation counter (Beckman Coulter Inc., Mississauga, ON).

2.5. Cell proliferation assay

Breast cancer cells were labeled with 2 μM Oregon Green 488 dye in serum-free DMEM for 1 h at 37 °C in a humidified 10% CO_2 incubator, then washed and resuspended in fully supplemented DMEM. At this time, non-proliferating control cells were collected and stored at 4 °C in 1% PFA (w/v) in PBS. Cells were seeded in 6-well flat bottom plates at 1.5×10^5 cells/well or 5×10^4 cells/well for 24 h and 72 h culture, respectively, in the absence or presence of apigenin. Cells were then collected, fixed in 1% PFA, and analyzed by flow cytometry. The number of cell divisions was calculated on the basis of mean fluorescence intensity of Oregon Green 488-stained cell samples and normalized to the medium control.

2.6. Cell cycle analysis

Following 24 h culture in the absence or presence of apigenin, breast cancer cells were collected, washed and resuspended in ice-cold PBS, and fixed by slowly adding ice-cold 70% ethanol with simultaneous vortexing. Samples were then stored at −20 °C for 24 h, washed, and resuspended in cell cycle analysis solution (0.2 mg/ml DNase-free RNase A, 0.02 mg/ml PI, and 0.1% Triton X-100 in PBS). After 30 min incubation in the dark at room temperature, samples were analyzed by flow cytometry and the percentage of cells in each stage of the cell cycle was determined using ModFit LT software (Verity Software House, Topsham, ME).

2.7. ROS measurement

Breast cancer cells were cultured for 6, 24, or 48 h in the absence or presence of apigenin. Cells were then harvested using TrypLE, washed, and resuspended in DMEM containing 10 μM DCF-DA. After incubation for 20 min at 37 °C, cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences, Mississauga, ON) to measure ROS production on the basis of median fluorescence intensity of cell samples.

2.8. Western blot analysis

Breast cancer cells were cultured for 48 h in the absence or presence of apigenin. In some experiments, GSH at a final concentration of 10 mM was also added to culture 30 min prior to treatment with apigenin. At the end of culture cells were harvested, washed with ice-cold PBS, and resuspended in 40 μl lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 50 mM Na_2PO_4, 5 mM EDTA, 5 mM EGTA, 0.25% w/v SDS, 0.1% v/v Nonidet P-40 at a pH of 7.5) containing protease inhibitors (5 μg/ml leupeptin, 5 μg/ml pepstatin, 10 μg/ml aprotinin, 100 μM sodium