Polyphenols, isothiocyanates, and carotenoid derivatives enhance estrogenic activity in bone cells but inhibit it in breast cancer cells

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INCIDENCE RATES of breast and endometrial cancer have been rising in the last decades, due to increased exposure to estrogens resulting from changes in lifestyle (19, 39). Thus, reduction of estrogen receptor activity is a major strategy for prevention of hormone-dependent malignancies. This preventive approach, however, must be implemented by taking into consideration that estrogens are beneficial to bone health. Women who enter menopause are at high risk for osteoporosis due to low blood levels of estrogens (42). Thus, since women today live longer after menopause than in a reproductive state, osteoporosis has become a serious threat to their health. To prevent hormone-dependent cancer without compromising bone health, specific estrogen receptor modulators (SERMs) have been developed (41). SERMs such as tamoxifen and raloxifene were successfully used for prevention of breast cancer in women who are at high risk for the disease and were also found to reduce the incidence of bone fractures (11, 54). However, for long-term prevention in the general population, dietary changes rather than drug application has long been considered to be a more sensible approach. In the present study, we address the question of whether plant-derived micronutrients possess a SERM-like activity, namely, whether they inhibit estrogenic activity in breast cancer cells while supporting estrogen activity in bone cells.

Epidemiological evidence suggests an inverse correlation between consumption of fruits and vegetables and the incidence of cancer. Three main groups of phytonutrients: carotenoids (15, 49), polyphenols (4, 56), and isothiocyanates (1, 46), have well-documented cancer-preventive activity. For example, in our previous studies we found that carotenoids, which are abundant in a fruit- and vegetable-rich diet, inhibited IGF-I-induced growth (34), estrogen-induced proliferation, and estrogenic activity in breast cancer cells (18). A similar inhibitory action was exerted by polyphenols such as carnosic acid, curcumin, and silibinin and the isothiocyanate sulforaphane (Atzmon A, Hirsch K, Salman H, Danilenko M, Levy J and Sharoni Y, unpublished results). However, to date, the molecular mechanisms underpinning the sustaining effect of phytonutrients on the beneficial estrogenic activity in bone cells remain to be elucidated. Interestingly, in experiments in rats and in epidemiological studies, a diet rich in fruit and vegetables was linked to bone health and prevention of osteoporosis (33, 35, 52). In addition, recent studies suggest a bone protective role of several carotenoids in aged human adults (26, 40, 43, 44). High intake or blood level of lycopene and other carotenoids were associated with improved bone mineral density (43), reduction in negative bone markers (40), and the risk of fractures (44). Further support for the beneficial activity of specific phytonutrients was found in studies using a large panel of bone cells (20, 32, 38) (for a recent review, see Ref. 51).

Phytochemicals, such as polyphenols, carotenoids, and isothiocyanates, are responsible for the beneficial effects on bone health. Polyphenols, such as curcumin, inhibit estrogen activity in breast cancer cells and enhance estrogenic activity in bone cells. These effects are in agreement with the findings of previous studies (18). These phytochemicals thus represent a promising approach for prevention of hormone-dependent cancers while maintaining bone health. The potential protective role of these phytochemicals in bone health has been supported by epidemiological studies (33, 35, 52). In addition, recent studies have shown that phytochemicals, such as lycopene and lutein, are associated with increased bone mineral density (43), reduction in negative bone markers (40), and the risk of fractures (44).

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Materials and methods

Materials. Crystalline carnosic acid (93–97%) was purchased from Alexis Biochemicals (Lausanne, Switzerland) and crystalline curcumin (>95%) from Cayman Chemicals (Ann Arbor, MI). Diapocarotene-10,10′-dial and diapocarotene-6,14′-dial, synthetic carotenoid derivatives (>99% purity), were synthesized and provided by Dr. Hansgeorg Ernst (BASF, Ludwigshafen, Germany). We used these derivatives and not the intact carotenoids such as lycopene and β-carotene, because we have recently shown that the derivatives are the active molecules in carotenoid preparations that induce the Nrf2/EpRE/ARE transcription system (25). Sulforaphane was a gift from F-L. Chung (Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington, DC). Dimethyl sulfoxide (DMSO), tert-butyl-hydroquinone (tBHQ), resveratrol, and 17β-estradiol (estradiol) were purchased from Sigma Chemicals (Rehovot, Israel). MG-63 human osteosarcoma cells purchased from American Type Culture Collection (Manassas, VA) were stably transfected with expression vector for ERα (see below). All cell lines were between passages 2 and 9 after thawing. T47D and MG-63 ERα cells were grown in DMEM, and MC3T3-E1 cells were grown in α-MEM containing penicillin (100 U/ml), streptomycin (0.1 mg/ml), nystatin (12.5 µg/ml), and 10% FCS in a humidified atmosphere with 5% CO2. Human recombinant insulin (6 U/ml) was added to T47D culture medium. G418 (425 µg/ml) was added to MG-63 ERα medium. Prior to each experiment, T47D cells were depleted of steroid hormones by maintaining them for 5 days in phenol red-free DMEM supplemented prior to Nrf2 overexpression experiments, all cells were similarly depleted of steroid hormones. All the experiments were performed in phenol red-free DMEM supplemented with 3% DCC-FCS.

Cell treatment solutions. The carotenoid derivative diapocarotene-10,10′-dial was dissolved in THF and solubilized in cell culture medium as described previously (24). BHQ, carnosic acid, resveratrol, diapocarotene-6,14′-dial, and estradiol were dissolved in ethanol. Curcumin and sulforaphane were dissolved in DMSO. The final concentrations of the solvents in both the treatment and the control were 0.5% THF, 0.2% ethanol, and 0.1% DMSO. The vehicle did not affect the parameters measured in the present experiments. The concentrations of the compounds in the culture medium were BHQ 15 µM, carotenoid derivatives 2–4.5 µM, sulforaphane 5 µM, curcumin 2.5 µM, carnosic acid 10 µM, resveratrol 10 µM, and estradiol 1 nM. All procedures were performed under reduced lighting.

Transient transfection and reporter gene assay. MC3T3-E1, MG-63 ERα, and T47D cells were transfected using jetPEI reagent (Polyplus Transfection, Illkirch, France). Cells were transfected with 0.1 µg of pERE reporter plasmid and 0.05 µg (T47D and MC3T3-E1) or 0.15 µg (MG-63 ERα) of Renilla luciferase expression vector. For measurement of EpRE/ARE transactivation, 0.2 µg of EpRE/ARE reporter plasmid and 0.05 µg (T47D and MC3T3-E1) or 0.15 µg (MG-63 ERα) of Renilla luciferase expression vector were added. For this purpose, cells were seeded in 24-well plates (9 × 104 cells/well for T47D and 4 × 105 cells/well for bone cells) in phenol-free DMEM containing 3% DCC-FCS. One day later, cells were rinsed once with the appropriate culture medium followed by addition of 0.45 ml of medium and 50 µl of DNA mixed with jetPEI reagent at a charge ratio of 1:2 for T47D and MC3T3-E1 cells and 1:3 for MG-63 ERα cells. The cells were then incubated for 4–6 h at 37°C. Medium was replaced with one supplemented with 3% depleted DCC-FCS plus the test compounds, and cells were incubated for another 20 h. Cell extracts were prepared for luciferase reporter assay (Dual Luciferase Reporter Assay System, Promega) according to the manufacturer’s instructions. For Nrf2 overexpression, cells were transfected with 0.075 µg of the expression vector or the same amount of shNrf2 plasmid. For reduction of Nrf2 level, cells were transfected with 0.3 µg of shNrf2 or shcon. The transfection was carried out by electroporation according to the manufacturer’s instructions (NanoEnTek, Seoul, Korea). The electroporation was performed in one pulse of 20 ms at a voltage of 1,700 V.

Stable transfection. MG-63 ERα cells were obtained by transfecting 2 × 106 MG-63 cells in 100-mm plates by using jetPEI reagent, as described above, with 10 µg of an ERα expression vector in pDNA3. Cells were incubated in medium containing 10% FCS for 2 days and then trypsinized and replated at 1 × 105, 2 × 105, and 1 × 106 cells/100 mm plate in medium containing 850 µg/ml G418...
(Calbiochem, Germany). Clones were collected after a 4-wk period in the presence of G418.

**Real-time PCR.** Total RNA was extracted from cells with the YRB kit (RBC Bioscience, Chung Ho, Taiwan), and cDNA was prepared with M-MuLV Reverse Transcriptase (Promega) according to the manufacturer’s instructions, using oligo(dT) as a primer. ALP, OPG, and OCN mRNA was determined by quantitative real-time PCR, and the results were normalized by GAPDH or β-actin mRNA content. The following primers were used, with the annealing temperatures shown in parentheses: mOCN (54.5°C): F primer, GCTTAACCTGCTTGTG, R primer, TGCTGTACATCCATACCT; mOPG (61.5°C): F primer, CGAGGACCACAATGAACAAGTG, R primer, TGGCTGTGACATCCATACTT; mALP (61.2°C): F primer, CCGATGGCACACCTGCTT, R primer, GAGGCATACGCCATTG.

**Western blotting.** Cells (2 × 10⁶) were treated with phytonutrients for 4 h, washed twice with ice-cold PBS, and then scraped into ice-cold lysis buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 10% (vol/vol) glycerol, 1% (vol/vol) Triton X-100, 1 mM EGTA, 2 mM sodium orthovanadate, 20 mM sodium pyrophosphate, 50 mM NaF, 0.2 mM EDTA, and Complete protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany). The lysates were incubated for 10 min on ice and then centrifuged (1,500 g, 10 min, 4°C). The pellet, containing the nuclei, was washed with lysis buffer (1,500 g, 10 min, 4°C), dried, and dissolved in SDS loading buffer. The supernatant, containing the cytoplasm, was further centrifuged (20,000 g, 15 min, 4°C). Equal amounts of protein (25–80 μg) were used for Western blotting.

**Fig. 1.** Plant-derived micronutrients inhibit estrogen-induced reporter activity. **A**). Induction of ERE reporter activity by estradiol (E₂) (0.1 nM) was defined as 100%. Values of reporter activity are means ± SE of 3–13 experiments, each performed in triplicate. **B**. Effects of phytonutrients alone or together with indicated phytonutrients at the following concentrations: carotenoid derivatives 2–4.5 μM, sulforaphane (SFN) 5 μM, curcumin 2.5 μM, carnosic acid (CA) 10 μM, resveratrol 10 μM, and tert-buty1-hydroquinone (tBHQ) 15 μM. Induction of ERE reporter activity by estradiol treatment was 3.4-fold in MC3T3-E1 cells, 6.9-fold in MG-63 ERα cells, and 40.9-fold in T47D cells. Effect of estradiol (E₂) was defined as 100%. Values of reporter activity are means ± SE of 3–13 experiments, each performed in triplicate. *P < 0.05, **P < 0.005, ***P < 0.001, significantly different from E₂ alone (dashed line). **C**: effects of phytonutrients alone in the absence of E₂. These results (means ± SE of 4 experiments) are expressed as percent activity in the presence of E₂ alone to allow comparison to the results in A. Indicated phytonutrients did not significantly alter basal activity except that of resveratrol. **D**: effects of phytonutrients alone in the absence of E₂. These results (means ± SE of 3–4 experiments) are expressed as percent activity in the presence of E₂ alone to allow comparison to the results in A. Indicated phytonutrients did not significantly alter basal activity except that of resveratrol.
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μg) were analyzed by Western blotting with the following primary antibodies: Nrf2 (H-300, sc-13032), ERα (C-20, sc-543), lamin B (C-20, sc-6216), and tubulin (H-235, sc-9104) all of which were obtained from Santa Cruz Biotechnology, (Santa Cruz, CA). Protein abundance was quantitated by densitometric analysis using the ImageMaster VDS-CL imaging system (Amersham Pharmacia Biotech, Piscataway, NJ).

**ALP activity.** Cells (5 × 10^5) were seeded in 60-mm culture dishes. A day later, the medium was changed with one containing the various test compounds in the presence of 10 mM β-glycerophosphate for 6 h. The cell monolayer was gently washed twice with PBS. The cells were lysed with 1% Triton X-100 and the lysates centrifuged at 2,000 g for 5 min. The supernatant was used for the measurement of ALP activity by a BCAProtein assay kit (Pierce, Rockford, IL).

**Chromatin immunoprecipitation.** MG-63 ERα cells (3 × 10^6 in 100-mm plates) were treated for 4 h with 30 μM phlorizin and 90 min with estradiol. Formaldehyde was added directly to the medium to a final concentration of 1% for 10 min at room temperature for cross-linking which was stopped by 5 min incubation with 0.125 M glycine. The cells were then washed twice with ice-cold PBS and collected by scraping into ice-cold PBS supplemented with a protease inhibitor cocktail (Roche, Mannheim, Germany). After centrifugation, the cell pellets were resuspended in 0.3 ml of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-Cl, pH 8.1) supplemented with Complete protease inhibitor cocktail for 10 min on ice and sonicated eight times for 15 s in a Vibra-cell sonicator, (Sonics and Materials, Newtown, CT) followed by centrifugation (15,000 g, 7 min, room temperature). The supernatants were diluted 1:10 in chromatin immunoprecipitation (ChIP) dilution buffer (0.01% SDS, 1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-Cl, pH 8.1, 16.7 mM NaCl, and protease inhibitors); 150 μl from each sample was taken as input and precipitated by overnight incubation at −20°C with 0.1 volumes of 3 M Na-acetate, pH 5.2, and 2.5 volumes of ethanol using glycogen as carrier. Precipitates were washed with ice-cold 70% ethanol and dried. Samples were precleared by incubation with salmon sperm DNA-protein A agarose (Upstate Biotechnology, Lake Placid, NY) at 4°C for 30 min with agitation. The samples were subjected to immunoprecipitation (overnight at 4°C with agitation) with the antibodies to Nrf2 (sc-722, Santa Cruz Biotechnology) or normal rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Complexes were recovered by 2-h incubation with 35 μl of salmon sperm DNA-protein A agarose and centrifugation (2,000 g for 2 min at 4°C). Precipitates were serially washed with 1 ml of each of the following buffers: Low Salt Wash Buffer (20 mM Tris-Cl, pH 8.0, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, pH 8, 500 mM NaCl) and LiCl Wash Buffer (10 mM Tris-Cl, pH 8.0, 0.25 M LiCl, 1% NP-40, 1% Na-deoxycholate, 1 mM EDTA, pH 8.0) and two times with TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0). The immunocomplexes and the inputs were eluted by adding 100 μl of Chelex (Bio-Rad, Hercules, CA) and incubation at 95°C for 10 min, and then digestion by adding 1.5 μl of protease K (Fermentas, Lita) and incubation at 55°C for 30 min with rotation. The samples and the inputs were centrifuged, and PCR amplification was performed on the supernatants with ReadyMIX PCR master mix (ABgene, Surrey, UK) using ARE-NQO1 primers for 35 cycles at annealing Tm of 62°C: F 5’–GCAGTTTCTAAGAGCAGAACG–3’, R 5’–GTAGATTAGTCCTCACTCAGCCG–3’. Statistical analysis. All experiments were repeated at least three times. The significance of the differences between the means of the various subgroups was assessed by two-tailed Student’s t-test using the GraphPad Prism 3 program (GraphPad Software, San Diego, CA). P < 0.05 was considered statistically significant. Statistically significant differences among the multiple groups in Fig. 1, B and C, were tested by two-way ANOVA using GraphPad Prism 3.0. P < 0.05 was considered statistically significant.

**RESULTS**

**Opposing effect of phytonutrients on estrogen-induced transcription in breast cancer and bone-derived cells.** We compared the effects of different phytonutrients, such as carotenoid derivatives (diapocarotene-10,10’-dial and diapocarotene-6,14’-dial), an isothiocyanate (sulforaphane), polyphenols (carnosic acid, curcumin, and resveratrol), and the synthetic polyphenol tBHQ on estrogenic activity in breast cancer cells and osteoblast-like cells by using a reporter gene assay. To this end, ERE transactivation by estradiol was measured in T47D human breast
cancer cells and in two osteoblast-like cell lines, MC3T3-E1 rodent cells, which express endogenous ER, and MG-63 human cells stably transfected with ERα (MG-63 ERα cells). Similar to our previous findings (18), all the phytonutrients tested significantly repressed estradiol-induced ERE activation in breast cancer cells (Fig. 1A). In contrast, the same concentrations of these compounds did not affect or even potentiate estradiol-stimulated ERE activity in both bone-derived cell lines (Fig. 1A). Diapocarotene-6,14-dial and carnosic acid were particularly active in both bone cell lines, whereas sulforaphane stimulated estrogenic activity to a larger extent in MC3T3-E1 cells. The basal activity in the absence of estradiol was -2 and 30% in T47D and MC3T3 cells, respectively. All the phytonutrients except resveratrol had only minor, insignificant effects on the basal ERE activity (Fig. 1B). Resveratrol, which is known to possess low estrogenic activity (45), indeed increased ERE activity to 62 ± 9 and 68 ± 15% of that induced by 1 nM estradiol in T47D and MC3T3-E1 cells, respectively. In view of the estrogenic activity of resveratrol, we did not study its effects on estradiol-induced activity any further.

Since plasma levels of estrogens change continuously in the body during the ovulation period and can vary from 0.09 to 2 nM, the effects of some of the phytonutrients on ERE transactivation were further tested in bone-derived cells over a wide range of estradiol concentrations, including the physiological range (Fig. 1, C and D). At concentrations ranging between 0.001 and 10 nM, estradiol showed a biphasic effect on ERE transactivation with maximal stimulation at 1 nM. The increase of this activity by carnosic acid and sulforaphane was similar over the whole range of estradiol concentrations in MC3T3-E1 cells (Fig. 1C). Although the magnitude of the effects of these two phytonutrients was different in MG-63 ERα cells, it was similar for the various estradiol concentrations (Fig. 1D). For this reason, all other experiments were performed with 1 nM estradiol.

Phytonutrients enhance the expression and activity of estrogen-induced bone-forming genes. To determine whether the enhancement of ERE activity by the phytonutrients as observed in the reporter assay has functional significance in bone-derived cells, we measured their effects on the expression and activity of several estrogen-regulated bone forming genes (OCN, OPG, and ALP). In MC3T3-E1 cells, we compared the breast cancer cell line T47D and the bone cell line MC3T3-E1, both expressing endogenous ER, to combinations with the tested phytonutrients affected Runx2 expression (Fig. 2A).

Phytonutrients affect ERα levels and its cellular localization. Stimulation of estrogen-induced transcription and the increase in mRNA levels of estrogen-responsive genes in bone cells and inhibition of estrogen-dependent transcription in breast cancer cells observed above suggest a tissue-specific activity of the phytonutrients. To understand the mechanism involved in this differential activity of the phytonutrients on ERE transactivation, we tested whether the microenvironments affect ERα levels in the cytosolic and nuclear fractions in a contrasting manner in the cancer cells vs. the bone cells (Fig. 3). In these experiments, we compared the breast cancer cell line T47D and the bone cell line MC3T3-E1, both expressing endogenous ER, to

Fig. 3. CA and diapocarotene-6,14-dial (6,14′) elevate nuclear ERα level in the presence of E2 in MC3T3-E1 cells but reduce it in T47D cells. T47D (A) and MC3T3-E1 cells (B) were treated for 4 h with E2 and phytonutrients as indicated. Cytosolic and nuclear fractions were prepared as described in MATERIALS AND METHODS and analyzed by Western blotting for expression of ERα. Representative blot from 3 experiments is shown. In the image of the nuclear ERα blot (B), the two central lanes surrounded by the dotted lines are inserted from the image of a parallel blot run in the same experiment and captured at the same exposure. C: total ERα level was calculated as the sum of cytosolic and nuclear ERα levels measured by densitometry of blots such as in A and B, as described in MATERIALS AND METHODS. Results are expressed as percent untreated control, defined as 100%, and are means ± SE of 3 experiments. *P < 0.05, significantly different from untreated control; *P < 0.05, significantly different from estradiol alone.
avoid artifacts that might result from exogenous expression of the receptor. We found that ERα disappeared from the cytoplasm in the presence of estradiol but not the phytonutrients diapocarotene-6,14′-dial and carnosic acid. This result was similar in both T47D breast cancer cells and MC3T3-E1 bone cells. Estradiol or the phytonutrients alone did not significantly affect ERα level in the nuclei of breast cancer cells, but their combination reduced the nuclear ERα level. On the other hand, in bone cells there was some increase of nuclear ERα level with estradiol treatment relative to the control. Remarkably, the combination of phytonutrients with estradiol greatly increased the ERα level in the nuclei of bone cells. In breast cancer cells, estradiol alone did not significantly affect the total ERα levels calculated by densitometry, whereas the combinations of estradiol with phytonutrients markedly decreased the receptor content (Fig. 3C). In contrast, estradiol alone significantly reduced the total ERα levels in bone cells, whereas the addition of phytonutrients abolished this reduction. The opposite effects of the phytonutrients in breast cancer and bone cells on the levels of nuclear and total ERα can explain, in part, their beneficial effects in both types of cells.

Phytonutrients activate the Nrf2/ARE transcription system in bone cells. We have previously shown that various phytonutrients activate the EpRE/ARE transcription system in several types of cancer cells (7, 8, 25). In the present study, we found that, similar to their effect in T47D breast cancer cells, all the tested phytonutrients as well as tBHQ transactivated the EpRE/ARE-reporter gene in the two osteoblast-like cells (Fig. 4A). The similar activation of the Nrf2/ARE system in cancer and bone cells was also evident from Western blotting results, which showed that the phytonutrients upregulated Nrf2 levels in the nuclei (Fig. 4B) as well as in the cytoplasm (data not shown). Estradiol alone had no effect. When added together with the phytonutrients, estradiol did not affect Nrf2 nuclear level in the bone cells but partially reduced it in the cancer cells (Fig. 4B). Furthermore, using the ChIP assay, we found that in MG-63 ERα cells carnosic acid increased Nrf2 binding to the EpRE/ARE sequence in the promoter of the phase II enzyme, NQO1 (Fig. 4C), whereas estradiol was ineffective. It would seem that carnosic acid, and not estradiol, caused this effect, since no binding was detected in samples treated only with estradiol. No binding was found using IgG-nonspecific antibody, which indicates the specificity of the interaction between Nrf2 and the EpRE/ARE sequence. Taken together, all the results presented in Fig. 4 indicate that the Nrf2/ARE transcription system is activated by the phytonutrients in osteoblast-like cells. Thus, we checked whether this transcription system modulates estrogenic activity in these cells.

Phytonutrients modulate estrogenic activity partially through the Nrf2/ARE transcription system. To examine the involvement of the Nrf2/ARE transcription system in the activation of
estrogen signaling in bone cells, we increased and decreased the expression of Nrf2. MC3T3-E1 bone cells and T47D breast cancer cells were cotransfected with Nrf2 expression vector and ps2-ERE-luc reporter plasmid (Fig. 5). Control cells were transfected with the appropriate empty vector. As expected, ERE transactivation was significantly reduced by Nrf2 overexpression in the breast cancer cells (Fig. 5A). In contrast, ERE transactivation was elevated in the bone cells (Fig. 5B). In the latter cells, estradiol, as well as diapocarotene-6,14'-dial, sulforaphane, and carnosic acid upregulated ERE-induced transcription regardless of Nrf2 overexpression (Fig. 5B).

To reduce the level of Nrf2 in MG-63 ERα bone cells, we transiently transfected the cells with shNrf2, which resulted in an ~80% reduction in Nrf2 levels (Western blotting, not shown). As expected, shNrf2 almost completely abolished EpRE/ARE transactivation (Fig. 6A). Cotransfection of shNrf2 with ERE reporter plasmid did not alter basal and estrogen-induced transcription but significantly inhibited the activation of estrogen-induced transcription by tBHQ, sulforaphane, and carnosic acid (Fig. 6B).

**DISCUSSION**

The main finding of this study is that phytonutrients, which were previously shown to inhibit estrogen signaling in breast cancer cells, do not inhibit and may even stimulate estrogen activity in bone cells. Interestingly, two bone cell lines, one expressing an endogenous ER and the other transfected with an ERα expression vector, exhibited similar responses to phytonutrients, suggesting that these responses are probably related to modulation of ER activity rather than to ER expression patterns, which may differ between endogenous and exogenous receptors. The opposite effects of phytonutrients on ERE activity in bone and cancer cells have not been reported. Interestingly, they are similar to those exhibited by known SERMs (41, 54). It is noteworthy that the molecular structure of these phytonutrients, especially that of the lycopene derivatives, e.g., diapocarotene-6,14'-dial, is different from that of known SERMs or estrogens, implying that the phytonutrients do not act by binding to ER. Resveratrol is the only compound, tested here, that is known to directly activate ER (45), and indeed, this compound induced ERE transactivation in the absence of estradiol in both the bone and the breast cancer cells. Of note, resveratrol did not increase further ERE activity in the presence of estradiol in bone cells and inhibited the effect of estradiol in breast cancer cells, an effect similar to the action of SERMs.

The phytonutrient concentrations used in the study are close to those that can be achieved by diet (14, 21, 31). The stimulation of estrogenic activity by some phytonutrients in bone cells, as analyzed by ERE reporter gene assay, was evident for a wide range of estradiol concentrations. The stimulation of this activity at the low estradiol concentrations that are generally found in women at menopause suggests that phytonutrients may positively regulate estrogenic activity in bone cells during and after reproductive age. Other reproductive hormones such as activin and inhibin regulate bone health (36) and are modified in menopause; thus, a putative effect of phytonutrients on their expression and activity should be considered in future studies.

The lack of inhibition of estrogenic activity in bone cells as opposed to cancer cells was evident not only in the reporter

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**Fig. 5.** Overexpression of Nrf2 decreases estrogen-induced ERE transactivation in breast cancer cells but enhances it in bone cells. T47D (A) and MC3T3-E1 cells (B) were depleted of steroids for 5 days and then cotransfected with ps2-ERE-luc reporter plasmid and 75 ng/well Nrf2 expression vector or a control vector. Cells were treated with diapocarotene-6,14'-dial (6,14'), SFN, or CA with or without E2. ERE reporter gene assay was performed as described in the legend to Fig. 1. Results are expressed as fold induction of untreated control cells. Dashed line indicates E2-induced transactivation in cells transfected with a control plasmid. Values of reporter activity are means ± SE of 2–4 experiments, each performed in triplicate. *P < 0.05, **P < 0.005, ***P < 0.001, significantly different from cells transfected with shcon plasmid.
gene assay but also when the effect of the carotenoid derivatives, curcumin, carnosic acid, and tBHQ on estrogen-induced transcription of bone forming genes was analyzed. Moreover, the expression of the genes osteocalcin, alkaline phosphatase, and osteoprotegerin and the activity of alkaline phosphatase, which are induced by estrogens (12, 30), increased in the presence of these dietary compounds. This rise in gene expression may result both from the estrogen-independent effect of the compounds as well as from the increase of estrogogenic activity. Indeed, an estrogen-independent increase in alkaline phosphatase activity after treatment with lycopene (38) and resveratrol (32) has been previously shown. Potentiation of transcription and activity of these genes by phytonutrients may indicate the enhancement of osteoblastic differentiation, ultimately resulting in improved bone formation. In addition to the effect on bone formation, the phytonutrients may impede excessive bone resorption via an increase in osteoprotegerin expression. This cytokine is known to inhibit excessive osteoclastic activity, which is prevalent during menopause when estrogen concentrations are low. If so, phytonutrients may also prevent osteoporosis acting similarly to SERMs such as raloxifene (29). We did not observe any effect of estradiol or phytonutrients on the expression of Runx2. The effect of estrogens on the level of this master bone regulator is controversial. McCarthy et al. (28) reported that estradiol enhanced Runx2 activity but did not change Runx2 protein levels in osteoblasts. In contrast, Taranta et al. (50) observed that estradiol and raloxifene increased Runx2 expression.

Alteration in nuclear and total ERα levels can explain how phytonutrients affect estrogentic activity in an opposite manner in bone cells vs. breast cancer cells. In breast cancer cells, carnosic acid and diapocarotene-6,14'-dial lowered ERα levels; however, in bone cells the phytonutrients increased nuclear ERα in the presence of estradiol and reversed the estradiol-induced decrease in total ERα. A plausible explanation is that the observed changes in ERα nuclear level and activity derive from tissue-specific degradation and covalent modifications of ERα. Consistent with this view, acetylation and phosphorylation were shown to modify ligand sensitivity, nuclear localization, and stability of ERα (10, 13, 23). It is an open question whether phytonutrients induce such modifications. Further studies are necessary to explore these alternatives.

Various phytonutrients have been shown to activate the Nrf2/ARE system; therefore, we hypothesized that Nrf2 is involved in the differential effects of phytonutrients on estrogen signaling in breast cancer vs. bone cells. An interaction between estradiol-bound ERα and Nrf2 has been shown previously in endometrial cancer cells (2, 57) resulting in repression of EpRE/ARE-regulated gene expression. This indicates that in these neoplastic cells the interaction occurs at the level of the transcription complex following activation by Nrf2. However, in our study in bone cells we did not find any changes in Nrf2 binding to EpRE/ARE sequences in the NQO1 gene promoter in the presence of estradiol, indicating that the inhibition of EpRE/ARE transcriptional activity by ER is tissue dependent. Moreover, we observed that Nrf2 overexpression elevated transcription via ERE in bone cells and inhibited it in breast cancer cells. Downregulation of Nrf2 by shRNA did not alter basal and estrogen-induced transcription, but phytonutrient activation of estrogen-induced transcription was inhibited in bone cells. Thus, in both cell types, activation of Nrf2 resulted in modulation of ERE-regulated transcription. The mechanism for the differential activity of Nrf2 in breast cancer and osteoblast cells remains to be elucidated.

Collectively, our results suggest a beneficial activity of the Nrf2 transcription factor in bone cells. These data are supported by a recent report by Xing et al. (55), who suggested that the supportive effect of a micronutrient (vitamin C) on bone formation is mediated by EpRE/ARE activation. Some studies that investigated the effects of nondietary stimuli on Nrf2/ARE activity have also suggested a positive role for Nrf2 in bone cells (6, 47); other studies, however, reported a deleterious effect of Nrf2 (3, 17, 53). Further research is necessary to determine the role of Nrf2/ARE activation by various factors in bone metabolism.

In conclusion, the current study indicates that phytonutrients can improve bone health by increasing estrogen-induced tran-
scription. This is in sharp contrast to their inhibitory action on estrogen signaling in breast cancer cells. We suggest two putative molecular mechanisms underpinning these opposite effects. The first proposes that phytonutrients affect ERα levels in the nuclei, and the second relies on the induction by these compounds of Nrf2 expression which, in turn, modulates estrogenic activity. Cumulatively, the present findings support the epidemiological evidence for the benefits of a fruit- and vegetable-rich diet and provide a tenable explanation for the paradox of the opposing activity of phytonutrients on estrogen signaling in different tissues.

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AUTHOR CONTRIBUTIONS


REFERENCES


