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

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Submitted 21 March 2011; accepted in final form 3 August 2011

Veprik A, Khanin M, Linnewiel-Hermoni K, Danilenko M, Levy J, Sharoni Y. Polyphenols, isothiocyanates, and carotenoids as estrogen agonists and antagonists: an overview. Am J Physiol Endocrinol Metab 303: E815–E824, 2012. First published August 30, 2011; doi:10.1152/ajpendo.00142.2011.—While exposure to estrogens is a major risk factor for breast and endometrial cancer, it well established that estrogens are beneficial for bone health. We have previously shown that carotenoids inhibit estrogen signaling in breast and endometrial cancer cells. The aim of this study was to compare the effects of various phytonutrients, (carotenoids derivatives, polyphenols, isothiocyanates) on estrogenic activity in breast cancer cells and osteoblast-like cells. All the tested phytonutrients inhibited estrogen response element (ERE) transactivation in breast cancer cells. In contrast, these compounds either did not affect or enhanced ERE activity and the expression of several bone-forming genes. These results were obtained using two osteoblast-like cell lines, MG-63 human osteosarcoma cells stably transfected with estrogen receptor-α (ERα) and MC3T3-E1 mouse calvaria-derived cells expressing endogenous ER. Phytonutrients-induced ERE inhibition in breast cancer cells, and its potentiation in osteoblast-like cells were associated both with a decrease and a rise in total and nuclear ERα levels, respectively. Phytonutrients activated the electrophile/antioxidant response element (EpRE/ARE) transcription system to various extents in both cancer and bone cell lines. Overexpression of Nrf2, the major EpRE/ARE activating transcription factor, mimicked the effects of phytonutrients, causing inhibition and enhancement of ERE transactivation in breast cancer cells and in osteoblast-like cells, respectively. Moreover, reduction in Nrf2 levels by RNAi led to a decrease in the phytonutrient potentiation of ERE activity transactivation in osteoblast-like cells. These findings suggest that the enhancement and inhibition of estrogen signaling by phytonutrients in bone-derived cells and breast cancer cells, respectively, is partially mediated by the activation of the Nrf2/ARE system.

Nrf2; estrogen receptor; osteoblasts; antioxidant response element; lycopene; curcumin

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 silybinin 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).

Estrogens upregulate osteoblast differentiation and transcription of genes that inhibit osteoclast differentiation. Moreover, they inhibit osteoclast acidification activity and expression of bone resorption genes (9). In addition, in osteoblasts, estrogens enhance transcription of many proteins that are important for bone formation, such as alkaline phosphatase (ALP), osteocalcin (OCN), and osteoprotegerin (OPG) (12, 30). ALP, an enzyme involved in the maintenance of plasma phosphate, is expressed in osteoblasts, serving as a marker of their differentiation (37). OCN is vital for bone mineralization.
control vector (shcon) were purchased from Sigma Chemicals. The following sequences were used: shNrf2, 5’-cgcggCTTCTCATGTGTGGTGAATTCATTATCAGTACATGAGGCTTTT-3’ and shcon, 5’-cgccAACAAGATGAGAGACCAACACTcgagTTTGAGTCTTCTAATCTTTGTTT-3’. Cell culture. The T47D human mammary cancer cell line was kindly provided by Dr. Iafa Keydar (Tel-Aviv University, Israel). MC3T3-E1, an osteoblast-like cell line derived from newborn mouse calvaria, was kindly provided by Dr. Itay Bab (Hebrew University, 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 used 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 μg/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 with 10% DCC-FCS. Prior to the 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). TBHQ, 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 presented experiments. The concentrations of the compounds in the culture medium were TBHQ 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 (Polypus Transfection, Illkirch, France). Cells were transfected with 0.1 μg of ER reporter plasmid and 0.05 μg of 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 of 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 × 104 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 pCDNA3. Cells were incubated in medium containing 10% FCS for 2 days and then trypsinized and replated at 1 × 104, 2 × 104, and 1 × 105 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, GCCCTACATATCCACGACG; R primer, CGCCCAATACGAC-

Western blotting. Cells (2 × 10⁶) were treated with phytonutrients for 4 h, washed twice with ice-cold PBS, and then scapped 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 DTT, 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 supernatant, containing the cytoplasm, was further centrifuged (20,000 g, 15 min, 4°C). Equal amounts of protein (25–80 µg) were diluted ninefold and mixed with 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-butyl-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, 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). B: effects of phytonutrients alone were tested 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. *P < 0.05, significantly different from basal activity except that of resveratrol. Gray area indicates physiological concentrations of E2. Results are means ± SE of 3–4 experiments, each performed in triplicate. Results for combinations of phytonutrients with E2 were significantly different from E2 alone. P < 0.05 by 2-way ANOVA.
PHYTONUTRIENTS ENHANCE ESTROGENIC ACTIVITY IN BONE CELLS

MATERIALS AND METHODS. Results are means ± SE of 3 (B) to 4 (A) experiments performed in duplicate. 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 cells.

Fig. 2. Plant-derived micronutrients upregulate transcription and activity of bone-forming genes. MC3T3-E1 (A) and MG-63 ERα cells (B) were treated for 4 days with indicated phytonutrients combined with E2. Total RNA was extracted from cells, and mRNA levels of indicated proteins were analyzed by real-time PCR as described in MATERIALS AND METHODS. Results are means ± SE of 3 (B) to 4 (A) experiments performed in duplicate. 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.

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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 ER\(\alpha\) (MG-63 E\(\alpha\)R 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 E\(\alpha\)R 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, our results showed that estradiol increased to various extents the expression of all three genes as determined by real-time PCR (Fig. 2A). The addition of diapocarotene-6,14′-dial, curcumin, carnosic acid, or tBHQ to the estradiol-treated cells further augmented this effect. Expression of one of these genes, OCN, was checked also in MG-63 E\(\alpha\)R cells but only with carnosic acid, and this polyphenol potentiated the estradiol-induced expression of this gene (Fig. 2B). In addition to mRNA levels, we also checked estradiol-induced stimulation of ALP activity, which was also enhanced by the phytonutrients, depending on the bone cell type (Fig. 2C). In MC3T3-E1 cells, a significant increase relative to the estradiol treatment alone, was obtained by diapocarotene-6,14′-dial, curcumin, carnosic acid, and tBHQ, whereas only sulforaphane enhanced the ALP activity in MG-63 E\(\alpha\)R cells. Runx2 is a major regulator of osteoblast differentiation and function; however, the role of estrogens on its expression is controversial (see DISCUSSION). In our experiments on MC3T3-E1 cells, neither estradiol nor its combinations with the tested phytonutrients affected Runx2 levels (Fig. 2A).

**Phytonutrients affect ER\(\alpha\) 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 micronutrients affect ER\(\alpha\) 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...
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 nucleus (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 inhibit 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

![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.](http://ajpendo.physiology.org/)}
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 estrogenic 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 estrogenic activity in an opposite manner in bone cells vs. breast cancer cells. In breast cancer cells, estrogens 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-
REFERENCES


