Research Article

Cancer Prevention Research

Berries and Ellagic Acid Prevent Estrogen-Induced Mammary Tumorigenesis by Modulating Enzymes of Estrogen Metabolism

Harini S. Aiyer[1] and Ramesh C. Gupta[1,2]

Abstract

To determine whether dietary berries and ellagic acid prevent 17β-estradiol (E_2)–induced mammary tumors by altering estrogen metabolism, we randomized August-Copenhagen Irish rats ($n = 6$ per group) into five groups: sham implant + control diet, E_2 implant + control diet (E_2-CD), E_2 + 2.5% black raspberry (E_2-BRB), E_2 + 2.5% blueberry (E_2-BB), and E_2 + 400 ppm ellagic acid (E_2-EA). Animals were euthanized at early (6 wk), intermediate (18 wk), and late (24 wk) phases of E_2 carcinogenesis, and the mammary tissue was analyzed for gene expression changes using quantitative real-time PCR. At 6 weeks, E_2 treatment caused a 48-fold increase in cytochrome P450 1A1 (*CYP1A1*; $P < 0.0001$), which was attenuated by both BRB and BB diets to 12- and 21-fold, respectively ($P < 0.001$). E_2 did not alter *CYP1B1* levels, but both berry and EA diets significantly suppressed it by 11- and 3.5-fold, respectively, from baseline ($P < 0.05$). There was a 5-fold increase in 17β-hydroxysteroid dehydrogenase 7 (*17βHSD7*), and this was moderately abrogated to ~2-fold by all supplementation ($P < 0.05$). At 18 weeks, *CYP1A1* was elevated by 15-fold in E_2-CD and only E_2-BB reduced this increase to 7-fold ($P < 0.05$). Catechol-*O*-methyltransferase expression was elevated 2-fold by E_2 treatment ($P < 0.05$), and all supplementation reversed this. At 24 weeks, *CYP1A1* expression was less pronounced but still high (8-fold) in E_2-treated rats. This increase was reduced to 3.2- and 4.6-fold by E_2-BRB and E_2-EA, respectively ($P < 0.05$), but not by E_2-BB. Supplementation did not alter the effect of E_2 on steroid receptors. The diets also significantly suppressed mammary tumor incidence (10-30%), volume (41-67%), and multiplicity (38-51%; $P < 0.05$). Berries may prevent mammary tumors by suppressing the levels of E_2-metabolizing enzymes during the early phase of E_2 carcinogenesis. *Cancer Prev Res; 3(6); 727–37. ©2010 AACR.*

Introduction

Breast cancer is the most diagnosed cancer among women in the United States and currently costs the American economy $85 billion in terms of value of life lost due to cancer mortality (1). The primary factors that determine mortality rate such as age, stage at diagnosis, and race/ethnicity are interlinked with another integral risk factor for breast cancer development—female reproductive hormone 17β-estradiol (E_2; ref. 2). Understanding the mechanism and prevention of E_2-induced breast cancer can lead to considerable long-term gains in both value of life for women as well as reduced health care costs in the United States. E_2 is a known, yet unavoidable risk factor for breast cancer. Women chronically exposed to even physiologic levels of this hormone can be at an increased risk to develop breast cancer (3).

Research suggests that *in situ* synthesis of estradiol may play a major role in the development of breast cancer, especially in postmenopausal women (4). Primary enzymes involved in *de novo* estradiol synthesis are aromatase, which converts androgen precursors to estrone, and 17β-hydroxysteroid dehydrogenase (17βHSD), which converts estrone (E_1) to estradiol (E_2; refs. 5, 6). Eight isozymes of 17βHSD have been identified thus far (7). The type 1 isozyme of 17βHSD, which converts estrone to estradiol, is found in both normal and malignant breast (8). The rodent homologue of this enzyme is 17βHSD type 7 (17βHSD7), also known as the prolactin receptor–associated protein (8, 9). This enzyme has high specificity for the conversion of E_1 to E_2 and is controlled by both prolactin and estrogen signaling pathways (9).

There are several phase I and II enzymes involved in the metabolism of E_2; of particular importance in the breast are cytochrome P450 1A1 (CYP1A1), CYP1B1, catechol-*O*-methyltransferase (COMT), UDP-glucuronosyltransferase (UGT), and glutathione *S*-transferase (GST). The phase I enzyme CYP1B1 has received wide attention due to its

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function in converting E_2 to 4-hyrdroxy estradiol ($4E_2$), a postulated potentially carcinogenic metabolite (10). In addition, breast tumors show high levels of both CYP1B1 and $4E_2$ (11, 12). Nevertheless, metabolites of CYP1A1 action, such as 2-hydroxy estrone, can produce stable DNA adducts, and inhibition of CYP1A1 metabolism reduces the formation of estrogen-induced kidney tumors in hamsters, suggesting that this pathway may also play a definitive role in estrogen carcinogenesis (13). The hydroxy metabolites of estradiol and estrone are conjugated for removal by several enzymes, including COMT, GST, and UGT (14, 15). The 2-hydroxy metabolites are better substrates for COMT (10), suggesting that CYP1A1 and COMT expression may be coupled. Polymorphisms in both phase I and II genes have been associated with a risk of breast cancer, indicating the importance of these enzymes in the production and removal of estradiol metabolites (4, 16). The estrogen metabolism pathway interacts with the estrogen signaling pathway. Hydroxy metabolites of estradiol, such as $4E_2$ and 2-hydroxy estradiol ($2E_2$), bind to estrogen receptors (ER) with varying affinities (17). Progesterone receptor (PGR) is upregulated by estrogen via ER signaling; hence, PGR expression is a downstream effect of ER activation (18). Thus, studying the expression of these genes provides some idea about control of estrogen metabolism in the mammary tissue.

Berries are an integral part of the Western cuisine and are also used in several other cuisines around the world. Blueberry and black raspberry have been commercially cultivated in the United States since the late 19th and early 20th century. They are also excellent sources of many vitamins, minerals, and cancer-preventing phytochemicals such as anthocyanins and ellagic acid (19). These berries vary significantly in both their phytochemical content and composition. Typically, black raspberry, which contains cyanidin as the primary anthocyanin component, is a richer source of ellagic acid than blueberry, whereas blueberry, a poor source of ellagic acid, contains five different anthocyanin pigments (20, 21). Both berries are high in antioxidant capacity and have shown cancer preventative effects (22, 23). We have previously shown that dietary berries (2.5%, w/w) and ellagic acid (400 ppm) can significantly inhibit the growth of E_2-induced mammary tumors in August-Copenhagen Irish (ACI) rats (24). Berries also prevented the pituitary-associated mortality (24) and reduced E_2-induced hepatic DNA adducts (25). However, the exact mechanisms by which they provide protection are not known. Berry phytochemicals such as anthocyanins and ellagic acid (and its metabolites urolithins A and B) show selective ER-modulating activity in some studies (26, 27). These phytochemicals can be absorbed into the systemic circulation in both humans and rodents and can be detected after ingestion at various levels (28–30). Thus, they may play a role in modulating estrogen metabolism in organs other than the gut, which was previously thought to be the primary target organ.

To determine whether berries and ellagic acid affect estrogen metabolism, we examined the regulation of gene expression of key enzymes involved in estrogen metabolism and signaling in the mammary tissue during the course of mammary tumorigenesis. Three time points—early (6 wk), intermediate (18 wk), and late (24 wk)—were chosen, and the expression of nine selective genes, three each involved in the phase I and II metabolism and estrogen signaling, were selected, and their relative gene expression changes were analyzed using quantitative real-time PCR (qRT-PCR). The genes tested were as follows: phase I metabolism, *17βHSD7*, *CYP1A1*, and *CYP1B1*; phase II metabolism, *COMT*, glutathione *S*-transferase A1 (*GSTA1*), and glutathione *S*-transferase M1 (*GSTM1*); and steroid signaling, *ERα*, *ERβ*, and *PGR*.

In a separate study, we also studied the effects of two doses (1% and 2.5%, w/w) of blueberry and black raspberry in an ACI rat model in which mammary tumors are induced by a lower dose of E_2 (9 mg) that significantly eliminates pituitary hyperplasia–induced mortality. Ellagic acid dose was maintained at 400 ppm, similar to the previous study, to provide a reference point (24). We measured the effect of dietary berries and ellagic acid on tumor incidence, latency, volume, and multiplicity to prove that berries are consistently effective in prevention of estrogen-induced mammary tumors.

Materials and Methods

Animals and treatment

Female ACI rats (7-8 wk old) were purchased from Harlan Sprague Dawley, housed under ambient conditions, and fed AIN-93M diet and water *ad libitum*. After a week of acclimation, 18 animals each were randomized into five groups. Two of the five groups received control diet and the other three received diets supplemented with 2.5% (w/w) dehydrated powdered blueberry, 2.5% (w/w) freeze-dried black raspberry, or 400 ppm ellagic acid. The sources, preparation, and caloric contents of these diets have been previously described in detail (24). After 2 weeks of prefeeding, each group received either sham implants or implants containing 27 mg E_2, as described (24). The animals were maintained on their respective diets throughout the study period; six animals from each group were euthanized at 6, 18, and 24 weeks after E_2 treatment by carbon dioxide asphyxiation; and mammary tissue was collected and frozen for further analysis.

In a separate second study, female ACI rats (5-6 wk old) were randomized into different groups (Table 1) and fed experimental diets for 2 weeks. Animals then received either a 1.2-cm silastic implant containing 9 mg E_2 as described (31) or sham implants and maintained on respective diets throughout the study. All diets were ordered from Harlan Teklad, Inc. The AIN-93M diet was supplemented with powdered berries (1% or 2.5%, w/w) or ellagic acid (400 ppm) and prepared as described earlier (24). Animals were weighed biweekly to track weight changes and disease progression. Mammary gland from this study was not used for RNA analysis.

Table 1. Comparison of organ weights and tumor indices between ACI rats fed control diet or diet supplemented with different doses of blueberry (BB), black raspberry (BRB), or 400 ppm ellagic acid

Group	Animal weight (g)	Liver (g)	Mammary (g)*	Pituitary (mg)	Tumor incidence (at 26 wk)†	Tumor volume (mm^3)†	Tumor multiplicity†
						(% reduction)	
Sham + control diet (n = 6)	182 ± 4 $P < 0.005$	4.6 ± 0.2 $P < 0.005$	3.4 ± 0.2 $P < 0.005$	9.6 ± 0.8 $P < 0.005$	NA	NA	NA
E_2 + control diet (n = 15)	204 ± 2	6.8 ± 0.2	4.8 ± 0.3	70 ± 4.5	100%	2,804 ± 547	11.7 ± 1.4
E_2 + 1% BB diet (n = 11)	207 ± 3	6.9 ± 0.2	5.4 ± 0.2	60 ± 7	100%	1,641 ± 405 –41%	11.4 ± 2.2
E_2 + 2.5% BB diet (n = 13)	203 ± 6	6.6 ± 0.3	5.7 ± 0.4	69 ± 16	69% $P < 0.05$	1,146 ± 276 NS –59%	7.2 ± 0.8 $P < 0.05$ –38%
E_2 + 1% BRB diet (n = 14)	201 ± 4	6.5 ± 0.2	6.0 ± 0.4	55 ± 9	81% $P < 0.05$	1,573 ± 403 NS –44%	6.6 ± 0.8 $P < 0.008$ –43.50%
E_2 + 2.5% BRB diet (n = 11)	210 ± 6	6.7 ± 0.3	7.0 ± 0.6 $P < 0.05$	39 ± 3 $P < 0.05$	87% $P < 0.05$	915 ± 250 $P < 0.05$ –67%	5.7 ± 1.0 $P < 0.007$ –51%
E_2 + ellagic acid diet (n = 11)	205 ± 6	6.5 ± 0.2	5.5 ± 0.3	57 ± 6	81% $P < 0.05$	983 ± 331 $P < 0.05$ –65%	6.9 ± 1.2 $P < 0.05$ –41%

NOTE: The data presented in this table are from a separate independent study carried out with a lower dose of the carcinogen E_2 than what was previously published (24). The key differences between the studies are described in detail in Materials and Methods as well as in Results. It was seen that berry/ellagic acid supplementation at the same dietary dose (2.5%/400 ppm) as before had a much higher preventive effect in animals where mammary tumors were induced with a lower dose of E_2. A lower dietary dose (1%) of black raspberries also had a significant effect.

Abbreviations: NA, not applicable; NS, not significant.

*Mammary glands were weighed after the removal of all grossly visible tumors. Typically, it was seen that the higher the number of tumors, the lower the mammary wet weight. Hence, there was a strong inverse correlation between tumor volume/multiplicity and mammary gland wet weight.

†Tumor incidence was compared using log-rank test, volume using one-way ANOVA, and multiplicity using Poisson regression as described in Materials and Methods. A P value of ≤ 0.05 was considered significant. All groups were compared to E_2 + control diet.

RNA isolation, reverse transcription, and qRT-PCR

RNA from whole mammary tissue was isolated using the Trizol method (Invitrogen), with modifications. Briefly, mammary tissue was suspended in Trizol at 4°C and homogenized with a handheld polytron at maximum speed. This homogenate was then passed through a syringe with a 22.5-gauge needle to ensure complete dissociation of the mammary tissue. The resultant tissue homogenate was sequentially extracted with chloroform, and the aqueous phase was precipitated using ice-cold isopropanol. The quality of the RNA was ascertained by gel electrophoresis and quantitated using NanoDrop (NanoDrop Technologies). RNA (100 ng) was reverse transcribed using the High-Capacity cDNA Archive kit (Applied Biosystems), and 3 ng of cDNA equivalent were used for PCR. These conditions were standardized to achieve consistent and reproducible results.

Primers for qRT-PCR were designed across exon boundary to avoid amplification of genomic DNA using Primer Express 3.0 software (Applied Biosystems) and synthesized by Integrated DNA Technologies, Inc. The sequences of the forward and reverse primers for each gene tested are listed in Table 2. The PCR amplification was done using Power SYBR Green PCR master mix (Applied Biosystems) and 500 nmol/L of forward and reverse primers for each gene, except *CYP1A1*, for which the final primer concentration was 125 nmol/L each. Quantitative PCR was done using a 7500 Fast Real-Time PCR system (Applied Biosystems). The PCR conditions were as follows: 50°C for 2 minutes, DNA polymerase activation at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. All gene analyses were done at least three times.

Analysis of gene expression

Gene expression analysis was done using the relative quantification ($\Delta\Delta C_T$) method as described (32). Each sample (refers to cDNA from individual animals) was

Table 2. Primer sequences used for qRT-PCR

Gene	Forward (5′-3′)	Reverse (5′-3′)
17βHSD	CTTTATCCTGATTCGGGAACTG	GTCCTCAAGACTGAAGTTAGAC
CYP1A1	TGGAGACCTTCCGACATTCAT	GGGATATAGAAGCCATTCAGACTTG
CYP1B1	AACCCAGAGGACTTTGATCCG	CGTCGTTTGCCCACTGAAAA
COMT	GGATGCAGTGATTCGGGAGTA	GCAGCGTAGTCAGGGTTCATCT
GSTA1	CCAGCCTTCTGACCTCTTTCC	TCTTCGATTTGTTTTGCATCCA
GSTM1	TCTTGACCAGTACCACATTTTTGAG	TCGAAAATATAGGTGTTGAGAGGTAGTG
ERα	GGCACATGAGTAACAAAGGCA	GGCATGAAGACGATGAGCAT
ERβ	CTCCTTTAGCGACCCATTGC	CTCCCACTAAGCTTCCTCTTCAGT
PGR	TCACAACGCTTCTATCAACTTACAAA	GGCAGCAATAACTTCAGACATCA
β-Actin	GCCAACCGTGAAAAGATGAC	ACCCTCATAGATGGGCACAG

NOTE: Primers were designed using Primer Express software across exon boundary for the following genes: *17βHSD7*, *CYP1A1*, *CYP1B1*, *COMT*, *GSTA1*, *GSTM1*, *ERα*, *ERβ*, and *PGR*.

analyzed in triplicate for each gene tested. ΔC_T was calculated as the difference between C_T of gene of interest (GOI) and the housekeeping gene *β-actin* ($\Delta C_T = C_{T\ GOI} - C_{T\ \beta\text{-actin}}$). One sample (sham treated) was chosen as the calibrator, and $\Delta\Delta C_T$ of all other samples was calculated using the formula $\Delta\Delta C_T = \Delta C_{T\ sample} - \Delta C_{T\ calibrator}$. A different calibrator sample (typically a sample from the sham-treated group) was chosen for the different time points (6, 18, and 24 wk), and fold change ($2^{-\Delta\Delta CT}$) in gene expression was calculated for all genes. The results are represented as relative fold change, which is the average fold change among the biological replicates ($n = 6$ per group) and represents the biological variation within a specific group.

Assessment of tumor indices for tumor study

In the second study, starting at 12 weeks after estrogen implantation, animals were palpated weekly for tumor appearance. The frequency of palpation was increased to twice a week, on appearance of the first tumor, to record tumor latency and incidence. Tumor incidence was calculated using the following formula: percentage tumor incidence = (number of tumor-bearing animals/total number of animals per group) × 100. The tumor incidence was considered 100% when all animals in a group had palpable tumors. The time between E_2 implants and the appearance of the first palpable tumor in any animal in a particular group was considered as the tumor latency for that group.

After 32 weeks of estrogen treatment, animals were euthanized and each animal was examined grossly for the presence of mammary tumors. Tumor volume was calculated as described (24). Liver, pituitary, and mammary glands were harvested and weighed. Representative tumors from each animal were analyzed by histopathology to confirm that they were mammary adenocarcinomas. All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Louisville.

Statistical analysis

Relative fold changes of gene expression in each group and tumor volumes were compared using one-way ANOVA, followed by a Tukey's multiple comparison post test. The difference in tumor incidence was assessed using the nonparametric log-rank test. All statistical analyses were done using the GraphPad Prism software (GraphPad Software), except for tumor multiplicity, which was compared in SAS version 8, using the Poisson Regression Model (SAS procedure PROC GENMOD). A P value of <0.05 was considered significant in all cases. The data are presented as mean ± SE.

Results

Two animal studies with slightly varying protocols are presented in this section. In the first study, ACI rats were implanted with 27 mg E_2 implants and euthanized at various time points (early, intermediate, and late) during the course of carcinogenesis. In this model, as published, all animals develop 100% mammary gland tumors by 24 weeks after implantation. Dietary berries and ellagic acid were very effective in reducing the tumor volume and multiplicity in the order 2.5% black raspberry > 400 ppm ellagic acid > 2.5% blueberry (24). However, a considerable shortcoming of this model has been its tendency to develop debilitating pituitary hyperplasia, as a response to the high E_2 dose, leading to significant morbidity and mortality (24, 31, 33). Several steps have been taken to correct this. Other investigators have taken a genomic approach and have developed a new strain of inbred rats that respond with reduced pituitary lactotroph hyperplasia on treatment with E_2 (34–36). Before this, we took a pharmacologic approach by reducing the E_2 dosage and showed that mammary tumors can be induced with 9 mg E_2, instead of 27 mg previously used, at a longer duration (32 instead of 24 wk) with essentially

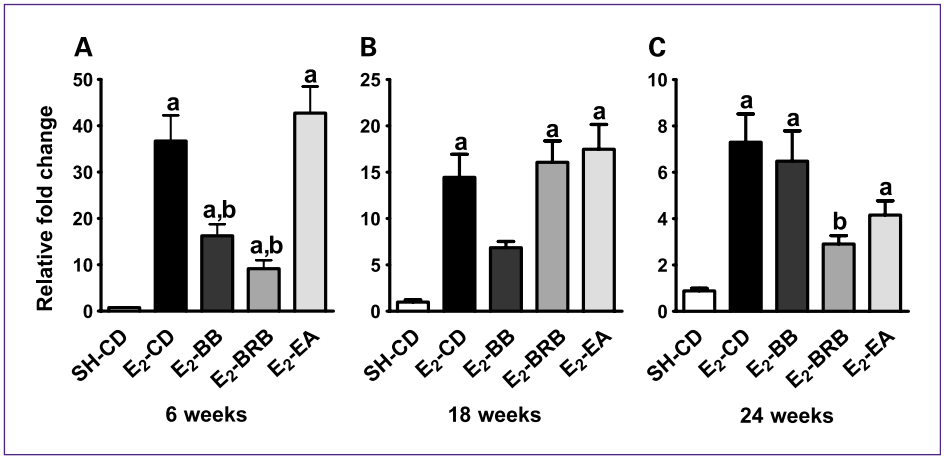

Fig. 1. A to C, effect of berry and ellagic acid diets on E_2-induced elevation in *CYP1A1* expression. ACI rats (n = 6) were treated with E_2 (27 mg) for 6 (A), 18 (B), or 24 (C) wk and fed either a control diet (E_2-CD) or diet supplemented with 2.5% (w/w) black raspberry (E_2-BRB), blueberry (E_2-BB), or E_2 + 400 ppm ellagic acid (E_2-EA). The whole mammary mRNA was analyzed for *CYP1A1* expression using qRT-PCR as described in Materials and Methods. The relative fold change was calculated using the $2^{-\Delta\Delta CT}$ method with a sample from SH-CD as the calibrator for each particular time point. a, $P < 0.0001$, significantly different from SH-CD; b, $P < 0.005$, significantly different from E_2-CD.

no mortality (31). This model with the reduced E_2 dose allows us to study the effects of different chemopreventive agents without the confounding mortality present in the previous model. The effect of dietary berries, even at doses lower (1%) than previously used (2.5%), on mammary tumor indices is presented here. Although two different protocols are presented, the assumption is that the basic mechanism of E_2 carcinogenesis is similar in both.

Dietary berries, but not ellagic acid, significantly reverse the E_2-induced increase in *CYP1A1* expression

In the early phases of treatment (6 wk), the strongest increase in expression after E_2 treatment occurred for *CYP1A1*. Compared with sham, E_2 treatment caused a 48-fold induction in *CYP1A1* expression (0.75 ± 0.09 versus 36.7 ± 5.5; $P < 0.0001$; Fig. 1A). This increase was significantly countered by both BB (16.3 ± 2.5; $P < 0.001$) and BRB (9.2 ± 1.8; $P < 0.001$) but not by ellagic acid (42.7 ± 5.7). The effect of E_2 on *CYP1A1* induction was somewhat blunted at 18 weeks to only 15-fold of sham levels (0.98 ± 0.2 versus 14.4 ± 2.5; $P < 0.01$; Fig. 1B). BB diet continued to counter this increase during the intermediate phase (6.8 ± 0.7), whereas BRB (16.1 ± 2.3) and ellagic acid (17.5 ± 2.7) did not show any effect. However, this trend was reversed during the late phase (24 wk). Although E_2 increased *CYP1A1* by only 8-fold compared with sham (0.9 ± 0.1 versus 7.3 ± 1.2; $P < 0.05$; Fig. 1C), both BRB (2.9 ± 0.4; $P < 0.05$) and

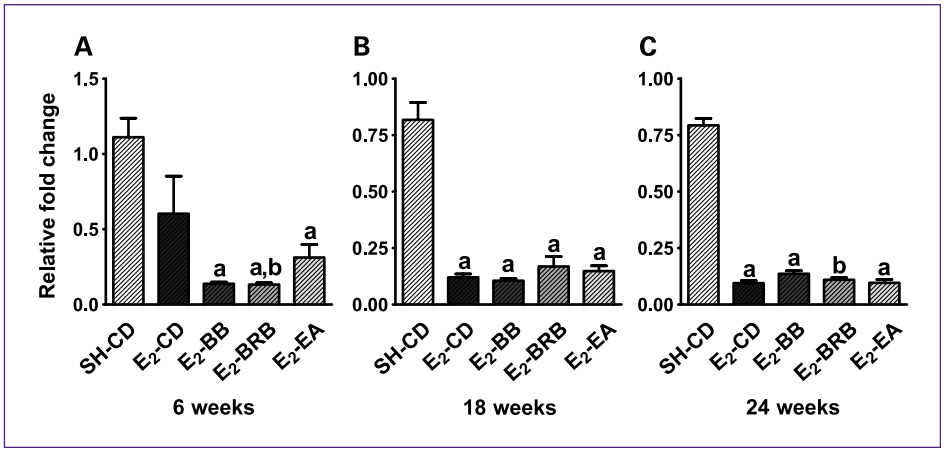

Fig. 2. A to C, effect of berry and ellagic acid diets on E_2-induced elevation in *CYP1B1* expression. ACI rats (n = 6) were treated with E_2 (27 mg) for 6 (A), 18 (B), or 24 (C) wk and fed either a control diet (E_2-CD) or diet supplemented with 2.5% (w/w) black raspberry (E_2-BRB), blueberry (E_2-BB), or 400 ppm ellagic acid (E_2-EA). The whole mammary mRNA was analyzed for *CYP1B1* expression using qRT-PCR as described in Materials and Methods. The relative fold change was calculated using the $2^{-\Delta\Delta CT}$ method with a sample from SH-CD as the calibrator for each particular time point. a, $P < 0.01$, significantly different from SH-CD; b, $P < 0.01$, significantly different from E_2-CD.

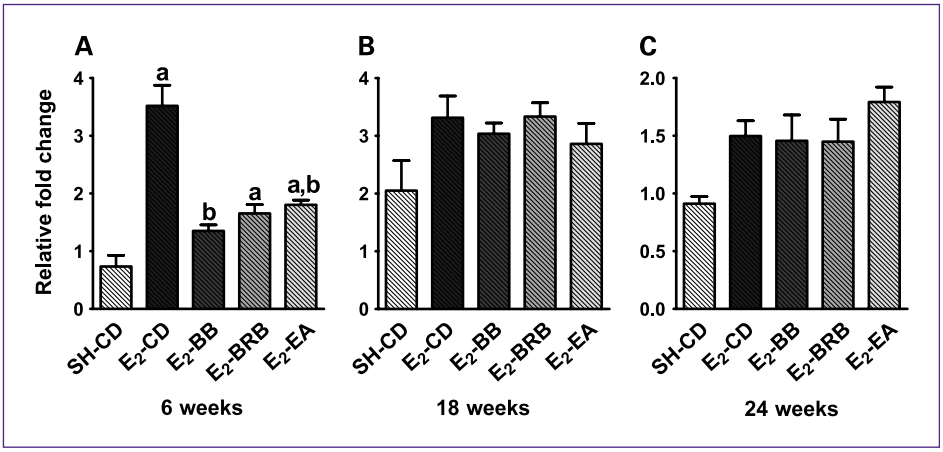

Fig. 3. A to C, effect of berry and ellagic acid diets on E_2-induced elevation in *17βHSD7* expression. ACI rats (n = 6) were treated with E_2 (27 mg) for 6 (A), 18 (B), or 24 (C) wk and fed either a control diet (E_2-CD) or diet supplemented with 2.5% (w/w) black raspberry (E_2-BRB), blueberry (E_2-BB), or 400 ppm ellagic acid (E_2-EA). The whole mammary mRNA was analyzed for *17βHSD7* expression using qRT-PCR as described in Materials and Methods. The relative fold change was calculated using the $2^{-\Delta\Delta CT}$ method with a sample from SH-CD as the calibrator for each particular time point. a, $P < 0.05$, significantly different from SH-CD; b, $P < 0.05$, significantly different from E_2-CD.

EA (4.2 ± 0.6) reduced this increase. BB diet showed only slight but insignificant reduction (Fig. 1C). In summary, E_2 significantly boosts the levels of *CYP1A1* mRNA during the early, middle, and late phases of E_2 carcinogenesis, with the effect plateauing over the time course. Blueberry is highly effective during the early and intermediate phase, black raspberry during early and late phase, and ellagic acid only during the late phase in countering this E_2-induced increase.

Both dietary berries and ellagic acid significantly reduce the levels of *CYP1B1* during the early phase of carcinogenesis

The level of *CYP1B1* mRNA in the whole mammary gland was essentially unaltered after 6 weeks of E_2 treatment [sham implant + control diet (SH-CD): 1.1 ± 0.1 versus E_2 implant + control diet (E_2-CD): 0.6 ± 0.2; not significant; Fig. 2A]. However, all supplemented diets significantly reduced the levels of *CYP1B1* both from baseline and E_2 treatment. Both BB and BRB had similar effects and lowered *CYP1B1 levels* by up to 11-fold from SH-CD [E_2 + 2.5% blueberry (E_2-BB): 0.1 ± 0.01 and E_2 + 2.5% black raspberry (E_2-BRB): 0.1 ± 0.01; $P < 0.01$] and 6-fold from E_2-CD. Ellagic acid was less effective and caused a 4-fold decrease compared with baseline (0.3 ± 0.1; $P < 0.05$). During the intermediate and late phases, E_2 treatment itself caused a significant reduction in *CYP1B1* levels (Fig. 2B and C), and consequently, supplementation did not have any additional effect on this decrease. All E_2-treated groups regardless of the supplementation had

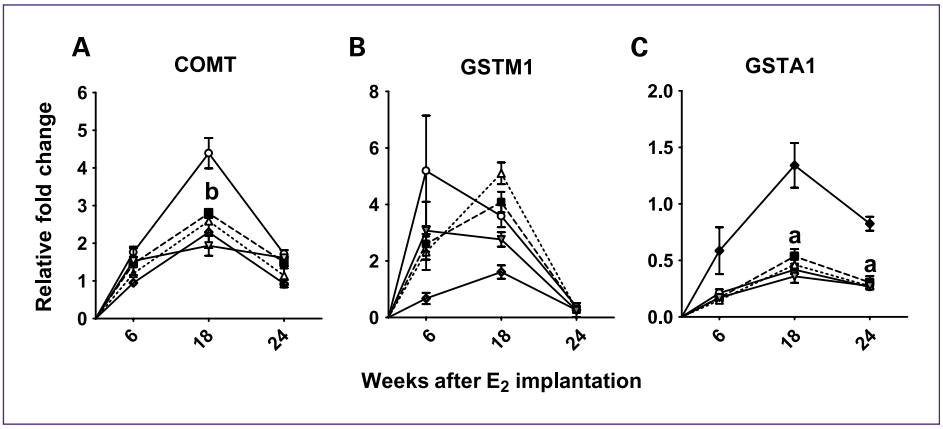

Fig. 4. A to C, effect of berry and ellagic acid diets on E_2-induced changes in phase II enzyme expression. ACI rats (n = 6) were treated with either sham implants or E_2 (27 mg) for 6, 18, or 24 wk and fed a control diet (SH-CD, ♦, solid line; E_2-CD, ○, solid line) or diet supplemented with 2.5% (w/w) black raspberry (E_2-BRB, Δ, dotted line), blueberry (E_2-BB, ■, dashed line), or 400 ppm ellagic acid (E_2-EA, ▼, solid line). The whole mammary mRNA was analyzed for *COMT* (A), *GSTM1* (B), and *GSTA1* (C) expression using qRT-PCR as described in Materials and Methods. The relative fold change was calculated using the $2^{-\Delta\Delta CT}$ method with a sample from SH-CD as the calibrator for each particular time point. a, $P < 0.05$, significantly different from SH-CD; b, $P < 0.05$, significantly different from E_2-CD.

similar CYP1B1 expressions at 18 and 24 weeks (Fig. 2B and C).

Dietary berries and ellagic acid counter the E_2-induced increase in *17βHSD7* expression at early phase of carcinogenesis

In the rat mammary, the enzyme *17βHSD7* plays an important role in *in situ* E_2 synthesis by converting E_1 to E_2. At 6 weeks, the expression of this enzyme increased by up to 5-fold after E_2 treatment (0.73 ± 0.2 versus 3.5 ± 0.4; $P < 0.01$; Fig. 3A). This increase was returned close to baseline levels effectively by all dietary supplementation: BB (1.3 ± 0.1; $P < 0.01$), BRB (1.6 ± 0.2; $P < 0.01$), and EA (1.5 ± 0.4; $P < 0.01$). This initial response to E_2 treatment did not persist during the intermediate and late phases of carcinogenesis (Fig. 3B and C). It seems that E_2-induced increase in *17βHSD7* expression is an early phase phenomenon and is countered effectively by berries and ellagic acid also during the early phase. The expression of another enzyme involved in *in situ* E_2 synthesis, aromatase, was undetectable in ACI rat mammary (data not shown).

Rats supplemented with berries and ellagic acid show significantly smaller induction in *COMT* levels during the intermediate phase of tumorigenesis

An important enzyme involved in the removal of harmful E_2 metabolites is *COMT*. Unlike the phase I enzymes, *COMT* is not induced during the early phase. Instead, there is a 2-fold increase in *COMT* expression during the intermediate phase (SH-CD: 2.3 ± 0.4 versus E_2-CD: 4.4 ± 0.4; $P < 0.05$; Fig. 4A). This increase is not seen in any of the supplemented groups (Fig. 4A), with BB (2.8 ± 0.1), BRB (2.6 ± 0.1), and EA (1.9 ± 0.3) essentially showing expression levels close to baseline. Two other phase II enzymes were analyzed: *GSTM1* was not altered by either E_2 treatment or supplementation (Fig. 4B), whereas *GSTA1* levels were found to be downregulated after estrogen treatment by up to 3-fold ($P < 0.05$) with no effect of intervention (Fig. 4C).

Berries or ellagic acid does not alter steroid receptors

The effect of E_2 treatment on classic ER pathway was analyzed by studying the expression of *ERα*, *ERβ*, and *PGR*, a downstream gene of *ERα* activation. As expected, E_2 treatment had a remarkable downregulatory effect on *ERα* expression at all time points (Fig. 5A). On the other hand, it had no effect on *ERβ* expression (Fig. 5B). *PGR* levels were significantly elevated at 6 weeks, suggesting activation of classic ER signaling (Fig. 5C). However, this increase was not sustained and fell to moderate levels during the intermediate phase and was very similar to sham treatment by the end of the study (Fig. 5C).

Effect of berry or ellagic acid supplementation on pituitary wet weight

E_2 treatment caused an increase in liver, mammary, and pituitary wet weights (Table 1). Compared with sham-treated animals, the most significant increase was seen in E_2-treated animals on control diet for pituitary weight, with >7-fold increase ($P < 0.005$). Blueberry diet at neither dose affected this increase. However, in animals fed black raspberry, both doses inhibited this E_2-induced increase in pituitary wet weight. The 2.5% dose reduced the weight by 44% compared with E_2-CD ($P < 0.05$) and 1% dose by 21% (not significant). Ellagic acid had the same effect as 1% BRB (21%; not significant).

Diets supplemented with black raspberry, blueberry, or ellagic acid significantly reduce tumor incidence

None of the sham-treated animals had any palpable or gross tumors. In animals fed the control diet, the first

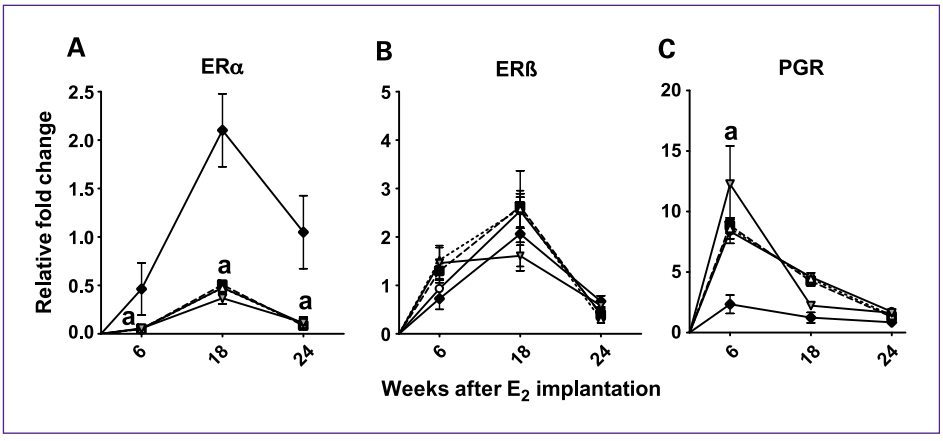

Fig. 5. A to C, effect of berry and ellagic acid diets on E_2-induced changes in steroid receptor expression. ACI rats ($n = 6$) were treated with either sham implants or E_2 (27 mg) for 6, 18, or 24 wk and fed a control diet (SH-CD, ♦, solid line; E_2-CD, ○, solid line) or diet supplemented with 2.5% (w/w) black raspberry (E_2-BRB, Δ, dotted line), blueberry (E_2-BB, ■, dashed line), or 400 ppm ellagic acid (E_2-EA, ▼, solid line). The whole mammary mRNA was analyzed for *ERα* (A), *ERβ* (B), and *PGR* (C) expression using qRT-PCR as described in Materials and Methods. The relative fold change was calculated using the $2^{-\Delta\Delta CT}$ method with a sample from SH-CD as the calibrator for each particular time point. a, $P < 0.01$, significantly different from SH-CD; b, $P < 0.01$, significantly different from E_2-CD.

palpable tumor appeared at 18 weeks of E_2 treatment (1 of 20 animals; 5% incidence). There was 50% incidence at just 20 weeks of treatment (10 of 20), and the linear trend continued until all animals in this group had palpable tumors by 26 weeks of treatment (Table 1). Although palpable mammary tumors were seen in animals fed 1% blueberry and 1% and 2.5% black raspberry diets (1 of 16; 6.25% for each group; Table 1), the incidence curves for all intervention groups, except 1% blueberry, were significantly different from the control diet (Table 1; $P < 0.05$, log-rank test). Blueberry at 1% did not affect tumor incidence (Table 1). However, at 2.5%, it had a highly significant effect and resulted in the lowest tumor incidence at 26 weeks (11 of 16; 69%; Table 1). Black raspberry at both 1% and 2.5% dose significantly shifted the tumor incidence curve to the right, resulting in 81% (13 of 16) and 87% (14 of 16) incidence at 26 weeks, respectively (Table 1). Ellagic acid (400 ppm) also had similar effects and significantly reduced incidence to 81% at 26 weeks (Table 1). The E_2 treatment was continued until 32 weeks.

Effect of supplemented diets on tumor volume and multiplicity

Tumor indices were measured for each animal and are represented as mean ± SE (Table 1). In animals fed control diet, the tumor volume and multiplicity were 2,804 ± 547 mm^3 and 11.7 ± 1.4, respectively. Tumor volume was reduced by all interventions from significant effects of 2.5% berry (59% for BB and 67% for BRB; $P < 0.05$) and ellagic acid diet (65%; $P < 0.05$) to marginal effects of 1% berry diets (41% for BB and 44% for BRB). The highest reduction in tumor multiplicity was achieved by 2.5% BRB (51%; $P < 0.007$), followed by 1% BRB (43%; $P < 0.008$), ellagic acid (41%; $P < 0.05$), and 2.5% BB (38%; $P < 0.05$); 1% BB did not affect tumor multiplicity.

Discussion

The results presented show that one of the main mechanisms by which berries and ellagic acid inhibit mammary tumors is by decreasing the levels of enzymes that can produce harmful E_2 metabolites. Our time course analysis also suggests that this inhibition occurs mostly during the early phases of carcinogenesis. Further, data from our tumor study show that berries consistently inhibit E_2-induced mammary tumors and black raspberry even at 1% dose shows significant chemopreventive efficacy.

The current study is the first to show that E_2 significantly affects the expression levels of enzymes that are involved in E_2 metabolism in the ACI rat. Previously published reports show that agents that alter E_2 metabolism and reduce oxidative stress can cause a reduction in mammary tumors in ACI rats (37, 38). Further, this is also the first report to show that berries and ellagic acid significantly reverse the effect of E_2 on these enzymes, thus potentially affecting the levels of harmful E_2 metabolites in the ACI rat mammary. Another significant finding is the analysis of gene expression changes throughout the course of carcinogenesis. The E_2-induced animal model varies vastly from the classic 7,12-dimethylbenz(a)anthracene–induced mammary tumor model in that the estrogen treatment is continuous, albeit in much lower doses. Thus, the conventional model of initiation, promotion, and progression does not apply. However, from the results presented, it seems that most of the detrimental effects of E_2 occur during the early phase and seem to level off during the later phases.

In this study, we show that E_2 significantly and consistently elevates *CYP1A1* expression to various levels throughout the carcinogenesis. *CYP1A1* is primarily known to catalyze the conversion of E_2 to the less harmful metabolite $2E_2$. Nevertheless, 15% to 20% of the E_2 metabolite produced by *CYP1A1* is $4E_2$ (10, 39). Mense and coworkers (37) have shown that E_2 treatment causes a higher ratio of $4E_2/2E_2$ in the ACI rat mammary. However, these studies were done using microsomes isolated from whole mammary and these investigators did not show exactly which enzymes are responsible. It is not clear whether the significant elevation of *CYP1A1* in our study actually leads to increased production of $4E_2$. Efforts are currently under way in our laboratory to identify the different metabolites using mass spectrometry. Future studies are planned to study the effect of E_2 with and without supplementation on the levels of various E_2 metabolites.

A surprising finding of this study was the effect of E_2 on *CYP1B1* levels, especially during the intermediate and late phases. It is well documented that the primary *CYP1B1* metabolite $4E_2$ is more harmful with respect to mammary tumorigenesis (40). To this date, only one study has shown a clear increase in $4E_2/2E_2$ ratio in the ACI rat mammary (37). However, no study has as yet shown a clear increase in CYP1B1 levels after E_2 treatment in ACI rats. E_2-treated mammary largely consists of proliferating cells of epithelial origin, whereas sham-treated tissue consists of a much higher percentage of stromal cells (31, 38). It is reported that CYP1B1 expression is constitutively higher in the rat mammary stroma, whereas CYP1A1 can be induced by estrogenic agents only in the epithelial cells (41). Thus, differences in the cell composition between sham and treated rats may potentially confound the results, as these analyses were done from total tissue RNA. Thus, the higher *CYP1B1* in untreated animals reflects the constitutive expression in the stromal compartment, whereas *CYP1A1* is upregulated by estrogen predominantly in epithelial cells and is thus increased by >40-fold.

Regardless of the effect of E_2, berries and ellagic acid significantly reduce the levels of both *CYP1A1* and *CYP1B1* expression at 6 weeks. The single most significant finding of this report is that berries and ellagic acid cause a net reduction in the expression of phase I enzymes responsible for converting E_2 to harmful metabolites, which in turn may lead to a net reduction in metabolites themselves, especially in the early stages. This is substantiated by the effect of both berries and ellagic acid on *COMT* expression at 18 weeks. The significant reduction in the *COMT* expression may be due to the constant suppression in the production of catechol estrogen metabolites by sustained

downregulation of *CYP1A1* and to a lesser extent of *CYP1B1*. It remains to be seen if the actual levels of E_2 metabolites are lower in animals fed berry and ellagic acid diets. Ellagic acid does not alter *CYP1A1* expression, suggesting that it differs from other berry phytochemicals (anthocyanins) in its mechanism of action. Previous reports suggest that ellagic acid does not alter the expression of hepatic CYP1A1 but inhibits its activity both *in vitro* and *in vivo* (42). It has also been shown that α-naphthoflavone, a CYP inhibitor, prevents mammary tumors in ACI rats (37).

Another interesting finding is the upregulation of *17βHSD7* by estradiol. This enzyme has high specificity for the conversion of estrone to estradiol in the mammary, suggesting that estradiol may influence *in situ* estrogen synthesis. However, 17βHSD expression is affected by both E_2 and prolactin in the rat corpus luteum (9, 43), and E_2 induces pituitary prolactinomas in this model (33). Thus, either E_2 directly influences the expression of *17βHSD7* or this may be a downstream effect of increased prolactin secretion. However, the expression of aromatase that forms estrone from androgen precursors is almost undetectable in the mammary tissue of the ACI rat (data not shown). Thus, the exact role of 17βHSD7 in *in situ* E_2 synthesis in ACI rat mammary remains to be shown.

Berries and ellagic acid also downregulate *17βHSD7*, which may further reduce *in situ* E_2 formation. In addition, *17βHSD7* is modulated by prolactin (9). In the current tumor study, we show that berries and ellagic acid significantly lower pituitary prolactinoma growth as evidenced by lower pituitary wet weight (Table 2). This finding suggests that berries regulate *17βHSD7* expression by possibly altering prolactin levels during the early phase or that they inhibit E_2-induced pituitary proliferation. There is support for the latter because both berries and ellagic acid significantly reduced pituitary-associated mortality in the previous tumor study (24).

In this study, with lower dose of E_2, we also show that dietary berries reduce tumor incidence, tumor multiplicity, and tumor volume in a dose-dependant manner. Black raspberry (2.5%, w/w) with the highest concentration of both anthocyanins and ellagic acid had the greatest effect on all three end points, followed by ellagic acid (400 ppm). The higher dose of blueberry (2.5%) had effects similar to that of low-dose black raspberry (1%). The lower dose of blueberry (1%) showed a marginal reduction in tumor volume but no effect on multiplicity or incidence (Table 1). Any confounding effect of caloric restriction on mammary tumor development can be safely ruled out, as no differences were seen in either the weight gain or the feed intake among control and supplemented diet fed groups (data not shown). Further, the supplemented diets were shown to be isocaloric to the AIN-93M diet (24). These results are highly consistent with our previous report in which 2.5% dietary berries or 400 ppm ellagic acid significantly diminished mammary tumors induced by high-dose E_2 regimen (24). However, in the current study, the 2.5% dose elicits a higher reduction of tumor volume and multiplicity than those observed in the previous model, suggesting that the toxicity due to the higher E_2 dose obscured the beneficial effects of supplementation.

Another important organ in this framework is the liver. The liver is responsible for the metabolism of circulating E_2, and it has been shown that metabolites of E_2 may play a significant role in mammary tumor development (13, 44). Mesia-Vela et al. (45) showed that altering the liver metabolism of E_2 significantly affected the mammary tumor development. We have previously shown that both dietary berries and ellagic acid significantly inhibit E_2-induced hepatic oxidative DNA adducts, showing that berries have a distinctive effect on the liver (25). It remains to be shown whether berries and berry phytochemicals cause a change of E_2 metabolism in the liver to bring about a change in mammary tumor development.

The differential effects of the two types of berries could be due to their distinctive anthocyanin profiles and contents. At a comparable dose, blueberry has only 2/3 the anthocyanin content and less than 1/20 of the ellagic acid content as that in black raspberry. Further, malvidin and delphinidin are the major anthocyanidins in blueberry followed by petunidin and peonidin, whereas black raspberry contains almost exclusively cyanidin (21, 46). Ellagic acid, the pure compound, consistently exhibits very similar effects regardless of the E_2 dosage. This suggests that, regardless of the E_2 dose used, similar mechanisms are involved in the prevention of E_2-induced mammary tumors by ellagic acid. Although the calculated levels of ellagic acid are eight times lower in 2.5% BRB diet (24), both ellagic acid and 2.5% BRB elicited very similar effects in reducing tumor volume, suggesting that whole-food source is more efficient than a purified component. This theory is supported by results from Wang and colleagues (47), who showed that the insoluble fraction of BRB containing just ellagitannins is as effective as either whole BRB or the anthocyanin-rich fraction in reducing esophageal tumors.

In summary, this is the first report to show both the changes in expression of E_2-metabolizing enzymes during the course of E_2 carcinogenesis in ACI rats and the effect of dietary berries/ellagic acid on the same. The changes that occur during the early phase in E_2-induced carcinogenesis are indicative of the efficacy of chemopreventive agents in reducing mammary tumor indices. CYP1A1 may play an important role in the E_2-induced tumorigenesis in the ACI rats. Dietary berries and ellagic acid cause a net reduction in the expression of phase I E_2-metabolizing enzymes. Black raspberry is thus far the most effective in reducing tumor incidence at 1% and 2.5%. It also has the greatest effect on phase I enzyme reduction at early phases. Blueberry, which has significantly lower levels of total phenolics than black raspberry, has much less effect on the enzyme expression, although the effect on tumor indices is more comparable, suggesting that different anthocyanidins (e.g., delphinidin) may be acting via alternative mechanism. Ellagic acid may act via mechanisms other than modulating CYP1A1 to significantly deter mammary tumor growth.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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