Prevention of Oxidative DNA Damage by Bioactive Berry Components

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The hormone 17β-estradiol (E2) causes oxidative DNA damage via redox cycling of its metabolites such as 4-hydroxy estradiol (4E2). In this study, ACI rats (8 wk old) were fed either AIN-93M diet or diets supplemented with 0.5% each of mixed berries (strawberry, blueberry, blackberry, and red and black raspberry), blueberry alone (BB; 2.5%), or ellagic acid (EA; 400 ppm) from 2 wk prior to and up to 12 wk of E2 treatment. The liver DNA was analyzed for the presence of 8-oxo-7,8-dihydroguanine (8-oxodG) and other polar adducts by 32P-postlabeling. Compared to sham treatment, E2 significantly increased the levels of both 8-oxodG and P-1 subgroup (259% and 214%, respectively; P < 0.001). BB diet also significantly reduced the levels of P-1, P-2, and PL-1 by 79, 63, 44, and 67%, respectively (P < 0.001). EA diet significantly reduced E2-induced levels of 8-oxodG, P-1, P-2, and PL-1 by 77, 43, and 68%, respectively (P < 0.001). Mixed berries were, however, ineffective. In addition, aqueous extracts of berries (2%) and EA (100 µM) were tested for their efficacy in diminishing oxidative DNA adducts induced by redox cycling of 4E2 catalyzed by copper chloride in vitro. EA was the most efficacious (90%), followed by extracts of red raspberry (70%), blueberry, and strawberry (50% each; P < 0.001).

INTRODUCTION

Breast cancer is the foremost cancer diagnosed among women worldwide (1). Over 95% of breast cancers diagnosed cannot be linked to a single causative factor and are considered sporadic in nature. It appears that several risk factors such as age at menarche, age at menopause, parity, family history, genetic polymorphisms, body weight, and other environmental factors such as diet, exercise, alcohol, and tobacco play a highly interactive role in the etiology of breast cancer. However, a common underlying factor that may influence all of these seemingly disparate risks is the hormonal status of the woman, especially that of 17β-estradiol (E2). The ages at menarche and menopause coherently determine the length of exposure (2). Family history has been linked to estrogen receptor status (3), polymorphisms of genes involved in estrogen metabolism (4), as well as to circulating hormone levels (5). Furthermore, interactions between diet, alcohol, and tobacco on metabolism of E2, which can affect the circulating hormone levels, have also been reported (6–8). The circulating levels of E2 itself is an independent risk factor (9). Thus, hormonal factors, especially of E2, play a major role in the etiology of breast cancer. E2 can cause both oxidative DNA damage (10) and reduced DNA repair leading to initiating mutational events (11). Its effect on promotion and progression of breast tumors has been extensively investigated [reviewed in (12,13)].

Estrogen-induced ACI rat mammary tumor model is a physiologically relevant model in which hormonal factors play a major role in mammary tumorigenesis. Akin to the human scenario, high levels of circulating E2 causes mammary tumors in these rats (14,15). Both progesterone and estradiol are required for the development of tumors (16). E2 causes DNA and epigenetic damage in the mammary (17,18), and blocking of E2 metabolizing enzymes or estrogen receptors abrogate the tumorigenesis (19). We have successfully utilized this animal model to study the chemopreventive efficacy of dietary berries and ellagic acid (EA) (20).

The hydroxylated E2 metabolite, 4-hydroxy estradiol (4E2), is known to be carcinogenic (10,21). The hepatic P450 enzymes, cytochrome P450 (CYP) 1A2 (CYP1A2) and CYP3A4 are primarily involved in the metabolism of E2 to its various phase 1 metabolites (22). Berries and berry components have been shown to affect enzymes involved in carcinogen metabolism in the liver (23–25). Liver is a suitable surrogate tissue to study the interplay of systemic E2 and bioactive dietary compounds, as it is a major metabolizing organ for both. Previously, we showed that dietary berries can significantly reduce baseline hepatic oxidative DNA damage in mice (24). To investigate whether berries and bioactive berry components can reduce E2-induced oxidative DNA damage, we used a 2-step approach. We analyzed the effect of 12-wk E2 treatment on the hepatic DNA damage in ACI rats fed either a control AIN-93M diet or diets supplemented with a mixture of berries (equal proportions of blueberry, blackberry, strawberry, red raspberry, and black raspberry), blueberry alone, or EA. Next, in an in vitro system, we studied the efficacy of aqueous berry extracts...
and EA to inhibit oxidative DNA damage induced by redox cycling of 4E2 and copper chloride (CuCl2). DNA adducts were analyzed by 32P-postlabeling thin-layer chromatography (TLC).

MATERIALS AND METHODS

Chemicals

EA was purchased from LKT laboratories (St. Paul, MN). Dimethyl sulfoxide (DMSO), CuCl2 and salmon testes (st)-DNA were purchased from Sigma Chemical Company (St. Louis, MO). 4E2 and E2 were purchased from Steraloids, Inc. (Newport, RI). Chemicals involved in 32P-postlabeling were purchased from sources described earlier (26). All chemicals used were >95% pure and were used without further purification. st-DNA was freed from contaminating RNA and protein prior to use as described previously (26,27).

Preparation of Aqueous Berry Extracts

Organic strawberry, blueberry, and red raspberry were purchased from the local cooperative grocery and processed as described (20). A total of 1 g of each berry powder was extracted with 10 ml of HPLC-grade water by shaking vigorously for 30 min followed by centrifugation at 10,000 g for 10 min. The supernatant was removed, and the residue was similarly extracted 2 more times. Pooled supernatants were concentrated to a volume of 1 ml using a lyophilizer (Savant SpeedVac, Thermo Fisher Scientific Inc., Pittsburgh, PA). This was considered as the 100% extract (equivalent to 1 g dry berry powder/ml).

In Vitro Reaction

st-DNA (300 µg/ml) in 10 mM Tris-hydrogen chloride, pH 7.4, was preincubated for 15 min at 37°C with vehicle (DMSO) alone, various berry extracts, or EA dissolved in DMSO at a final concentration of 2% vol/vol and 100 µM, respectively. Redox cycling was initiated by the addition of 4E2 (100 µM) in ethanol and CuCl2(100 µM) in water. After incubation at 37°C for 1 h, DNA was purified by solvent extraction and ethanol precipitation as described (26,28).

Diet Preparation

A standard AIN-93M powdered diet was ordered from Research Diets Inc. (New Brunswick, NJ). The berries were added to this at a dose of 2.5% wt/wt and EA at 400 ppm, mixed thoroughly in a Hobart mixer and stored at 4°C until use. For the mixed berry diet, 5 different types of dehydrated and powdered berries—blueberry, blackberry, strawberry, red raspberry, and black raspberry—were mixed in equal proportion so that the final diet contained 0.5% of each of these berries. The levels of EA and anthocyanins present in these diets were estimated based on the known concentrations of the same in various berries from previously published reports (29,30) and are shown in Table 1.

Animal Treatment

Female ACI rats (7-8 wk old) were purchased from Harlan-Sprague-Dawley, Inc. (Indianapolis, IN), housed under ambient conditions, and had access to food and water ad libitum. Animals were acclimated for 1 wk on AIN-93M diet prior to randomizing them into different groups. After feeding experimental diets for 2 wk, animals then received either a 3-cm silastic implant containing 27 mg E2 as described (14) or sham implants. The rats were euthanized after 12 wk of treatment, and the liver was snap frozen in liquid nitrogen for further analysis.

Analysis of DNA Adducts by 32P-Postlabeling

DNA from liver was isolated as described (26), and 14 µg was digested to 3′-monophosphates using micrococcal nuclease/spleen phosphodiesterase (Enzyme:DNA, 1:5, 5 h, 37°C). After removing 2 µg of digest for normal nucleotide analysis, 10 µg digest was enriched for polar adducts by treatment with nuclease P1 (Enzyme:DNA, 1:2.5, 1 h, 37°C). Remaining 2 µg of the digest was enriched for 8-oxo-7,8-dihydroguanine (8-oxodG) by polyethyleneimine (PEI)-cellulose TLC and 0.5–1 µg was labeled as described (31). The 5′-32P-labeling of both enriched DNA adducts and normal nucleotides were done in parallel by T4-polynucleotide kinase and molar excess of [γ-32P]adenosine triphosphate as described earlier (26,27). Labeled adducts were separated by 2-D PEI-cellulose TLC using 50 mM sodium phosphate, pH 6.0, and 1 M formic acid in the D1 direction. D2 was perpendicular to D1 using a solvent mixture of isopropanol:4M ammonium hydroxide:8M urea (3.3:1:1.6). Adducts with decreasing polarities in tissue DNA were eluted by increasing the sodium phosphate concentrations (50 mM–1,000 mM) in the presence of 1 M formic acid (D1) but maintaining the same D2 solvent (27). The adduct subgroups were classified based on their polarity (P) or lipophilicity (L), with the most polar subgroups named P-1 and the most lipophillic subgroups as L-1 and the adducts in between as PL-1, 2, etc. The enriched 8-oxodGp was labeled in parallel and chromatographed as described (31). Adducts and normal nucleotides were visualized using Packard InstantImager and were counted individually. Adduct levels were calculated as relative adduct labeling = [counts per minute (CPM) adducts/CPM normal nucleotides] × 1/dilution factor and are expressed as adducts per 10^6 nucleotides (in vivo adducts).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 4.00 for Windows, GraphPad Software (San Diego, CA). The results were compared using 1-way analysis of variance followed by either a Tukey’s t-test (in vivo adducts) or Dunnett’s multiple comparison test (in vitro adducts) as specified. A P value < 0.05 was considered significant.
TABLE 1
Estimated ellagic acid and anthocyanin contents of various diets

<table>
<thead>
<tr>
<th></th>
<th>Ellagic acid</th>
<th>Total Anthocyanin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control diet (AIN-93M)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2.5% Mixed berry diet</td>
<td>28.5</td>
<td>726</td>
</tr>
<tr>
<td>2.5% Blueberry diet</td>
<td>&lt;2.5</td>
<td>975</td>
</tr>
<tr>
<td>Ellagic acid diet</td>
<td>400</td>
<td>0</td>
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</table>

*a* Calculated from ellagic acid contents described in Daniel et al. (29).

*b* Anthocyanin contents described in Wu et al. (30).

RESULTS

Modulation of Estrogen-Induced Oxidative-DNA Damage (8-oxodG) by Dietary EA and Berries

All groups showed qualitatively similar patterns for 8-oxodG when analyzed by 2-D PEI-cellulose TLC (Fig. 1A). In sham-treated ACI rats, the baseline levels of endogenous 8-oxodG was 5,454 ± 1,238 per 10^9 nucleotides (Table 2). After 12 wk of E2 treatment, in animals fed control diet, this oxidative lesion was increased by 2.6-fold (Fig. A1 vs. A2; Fig. 1B). Dietary supplementation with EA protected significantly against E2-induced 8-oxodG levels by more than fivefold (Fig 1B; Table 2). This treatment also reduced 8-oxodG levels below baseline, but this change was not significant (Fig. 1B). Dietary berries did not significantly affect the levels 8-oxodG (Fig. 1B, Table 2).

Modulation of Novel Polar DNA Adducts by Dietary EA and Berries

The hepatic DNA of all groups of animals showed qualitatively similar patterns of uncharacterized polar adducts (Fig. 2). The baseline levels of different subgroups of adducts in the liver of sham-treated ACI rats fed control diet were P-1, 10,160 ± 729; P-2, 1,668 ± 121; PL-1, 3,459 ± 455; and PL-2, 555 ± 55 per 10^9 nucleotides (Table 2). L-1 adducts were too low to be quantified. The P-1 adduct levels significantly increased after 12-wk treatment with E2 implants, suggesting that the increase may be estrogen-induced (53%; *P* < 0.05; Fig. 3A). However, P-2, PL-1, and PL-2 adduct subgroups were unaffected (Table 2; Figs. 3B–3D).

Blueberry diet was highly effective in reducing the levels of P-1 (77%; *P* < 0.001), P-2 (43%; *P* < 0.001), and PL-1 (68%; *P* < 0.001) adduct subgroups. EA diet was highly significant in reducing the level of all subgroups of adducts except PL-2 (Table 2; Fig. 3). It significantly inhibited the formation of E2-induced P-1 adducts by 63% (*P* < 0.001; Fig. 3A) but showed only a moderately protective effect toward P-2 (44%; *P* < 0.001; Fig. 3B). Because the levels of PL-1 adducts were not induced by E2 treatment, EA showed a significant reduction of this subgroup even below baseline levels (67%; *P* < 0.001; Fig. 3C). It also showed a moderate but insignificant effect in reducing the PL-2 adduct levels (Fig. 3D, Table 2). Mixed berry diet did not affect the levels of any adduct subgroup (Fig. 3).
### Table 2

Modulation of E2-induced hepatic DNA adducts by dietary berries (2.5%, wt/wt) and ellagic acid (400 ppm)\(^a\)

<table>
<thead>
<tr>
<th>Group</th>
<th>P-1</th>
<th>P-2</th>
<th>PL-1</th>
<th>PL-2</th>
<th>8-oxodG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham + Control diet ((n = 2))</td>
<td>10,160 ± 729</td>
<td>1,668 ± 121</td>
<td>3459 ± 455</td>
<td>555 ± 55</td>
<td>5,454 ± 1238</td>
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<tr>
<td>E2 + Control diet ((n = 4))</td>
<td>21,774 ± 3116</td>
<td>1,853 ± 180</td>
<td>3625 ± 352</td>
<td>449 ± 45</td>
<td>14,158 ± 2522</td>
</tr>
<tr>
<td>E2 + Mixed berry diet ((n = 4))</td>
<td>16,820 ± 2080</td>
<td>1,751 ± 153</td>
<td>3126 ± 377</td>
<td>524 ± 48</td>
<td>12,050 ± 2840</td>
</tr>
<tr>
<td>E2 + Blueberry diet ((n = 4))</td>
<td>4,908 ± 1073</td>
<td>1,052 ± 76</td>
<td>1144 ± 167</td>
<td>514 ± 33</td>
<td>19,313 ± 550</td>
</tr>
<tr>
<td>E2 + Ellagic acid diet ((n = 4))</td>
<td>8,055 ± 1032</td>
<td>1,029 ± 103</td>
<td>1177 ± 220</td>
<td>298 ± 31</td>
<td>2,993 ± 1013</td>
</tr>
</tbody>
</table>

\(^a\)Adducts were separated by 2-directional polyethyleneimine thin-layer chromatography using solvent composition as described in Materials and Methods and visualized using a Packard InstantImager\(^\text{®}\). Each subgroup of adduct was individually measured and quantified using the formula relative adduct labeling = counts per minute (cpm) adducts/cpm normal nucleotides × 1/dilution factor as described (26). The values represented are the mean of 3 separate analyses. All groups were compared to E2 + control diet using 1-way analysis of variance followed by a Tukey’s post hoc test.

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**Modulation of 4E2/CuCl2-Induced Oxidative DNA Adducts by Aqueous Berry Extracts and EA**

Analysis of DNA damage, induced by redox cycling of 4E2 catalyzed by copper (Cu\(^{2+}\), revealed several uncharacterized oxidative adducts (Fig. 4A). These adducts were chromatographically similar to adducts generated by treatment of DNA with H\(_2\)O\(_2\)/CuCl\(_2\) (unpublished data). Neither 4E2 nor CuCl\(_2\) by themselves showed a significant increase in the baseline adduct levels (not shown). The levels of these polar adducts in the untreated st-DNA were low at baseline and increased significantly after treatment with 4E2 and CuCl\(_2\). The adduct levels were comparable to previously published levels (24).

Aqueous extracts of commonly consumed berries such as strawberry, blueberry, and red raspberry were tested for the inhibition of 4E2/CuCl2-induced DNA damage at 2% final con-
FIG. 4. Representative $^{32}$P-labeled maps of oxidative in vitro DNA adducts generated by copper chloride-catalyzed redox cycling of 4-hydroxy estradiol A: in the presence of vehicle control, B: aqueous red raspberry extract, or C: ellagic acid (EA) and D: the effect of various berry extracts (2%) and EA (100 µM) on adduct levels. Oxidative adducts (uncharacterized) were measured using $^{32}$P-postlabeling/thin-layer chromatography and are represented as mean ± SE of 4 replicates. The groups were compared to the vehicle control using 1-way analysis of variance followed by a Dunnett’s multiple comparison tests. Statistically significant differences are presented. SB, strawberry; BB, blueberry; RRB, red raspberry.

centration. Compared to the vehicle control, these extracts significantly inhibited oxidative-DNA damage from 50–70% ($P < 0.001$). Blueberry and strawberry extracts had comparable efficacies, and reduced DNA damage by 50% (Fig. 4D). Red raspberry was the most efficacious and showed a 70% inhibition in total adduct levels (Fig. 4B, 4D; $P < 0.001$). EA (100 µM final concentration) used as a positive control, showed the highest reduction (>90%; Fig. 4C, 4D) as previously reported (24).

**DISCUSSION**

The induction of primary mammary adenocarcinomas in ACI rats by exogenous estrogen treatment allows us to investigate several important aspects of estrogen-induced carcinogenesis. This includes the modulation of hepatic metabolism of estrogen and subsequent effects on mammary tumorigenesis. Kauffman’s laboratory (32,33) has demonstrated that alteration of hepatic metabolism of E2 significantly alters the mammary tumor incidence. Wilson and Reed (34) suggested that the species differences in susceptibility to estrogen-induced tumors may arise from distinctive hepatic metabolism of E2 to 4E2 in the ACI rat liver. The predominant hepatic P450 enzymes involved in the oxidative metabolism of E2 are CYP1A2 and 3A4 (35). Both these enzymes have been shown to consistently produce 4E2 as their metabolic byproduct in addition to the primary product 2E2 (22). 4E2 has been identified as the major carcinogenic metabolite of E2 and is capable of inducing oxidative DNA damage in vivo (10). It is possible to surmise that 4E2 may be involved in the E2-induced increase in oxidative hepatic DNA damage, reported in this study, due to the following reasons. We have previously shown that redox cycling of 4E2 catalyzed by CuCl2 can significantly induce 8-oxodG in vitro, and this induction is inhibited by EA starting at just 30 µM (24). Likewise, in vivo results presented in this report show that 8-oxodG can be induced up to 2.6-fold by E2 treatment, and this is significantly offset by the EA diet (Fig. 1B). As the novel subgroups of adducts quantified in this study are as yet uncharacterized, the mechanism of their formation is unclear. However, P-1 adducts are modulated in a fashion similar to 8-oxodG by both E2 and EA, suggesting that they may potentially arise via similar mechanisms. Dietary EA consistently reduces the levels of hepatic DNA damage both at baseline (24) and in the presence of E2 challenge (Figs. 1 and 3).

Aqueous extracts of different berries tested show inhibition of DNA damage, and the relative inhibition is directly associated with their total EA and anthocyanin contents (Table 3). Red raspberry, with the highest EA and moderate anthocyanin content, shows higher efficacy than strawberry, which has similar anthocyanin content but lower EA content (Table 3). On the other hand, blueberry, with the highest anthocyanin content, is as efficacious as strawberry. These results suggest that both EA and anthocyanins act additively and/or synergistically to counter E2-induced DNA damage in vitro. Both EA and anthocyanins are known antioxidants. Thus, in addition to EA, anthocyanins may play a significant role in the prevention of E2-induced mammary tumorigenesis (20). This is evident from the in vivo results presented wherein dietary blueberry significantly reduces the levels of P-1, P-2, and PL-1 adducts but had no effect on the 8-oxodG levels. In studies in which dietary

**TABLE 3**

<table>
<thead>
<tr>
<th></th>
<th>Ellagic acid</th>
<th>Total Anthocyanin</th>
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<tr>
<td>Red raspberry</td>
<td>1.5</td>
<td>9.2</td>
</tr>
<tr>
<td>Black raspberry</td>
<td>2.0</td>
<td>69</td>
</tr>
<tr>
<td>Blackberry</td>
<td>1.5</td>
<td>24</td>
</tr>
<tr>
<td>Strawberry</td>
<td>0.5</td>
<td>4.0</td>
</tr>
<tr>
<td>Blueberry</td>
<td>&lt;0.1</td>
<td>39</td>
</tr>
</tbody>
</table>

*Adapted from Daniel et al. (29) and Wu et al. (30).*
supplementation with anthocyanin-rich fruit juices was provided, 8-oxodG levels were not significantly affected, suggesting that anthocyanins may act via mechanisms other than reduction of 8-oxodG (36,37). Surprisingly, the mixed berry diet, which is high in both anthocyanin and EA content, did not show a significant effect in our study. The different berries mixed in this study have distinctly different anthocyanidin profiles. For example, blackberry and black and red raspberry are known to contain higher levels of cyanidin, strawberry has a higher content of pelargonidin, whereas all 5 anthocyanidins are evenly distributed in blueberry (30). However, when provided at only 0.5% dose, none of the anthocyanidin polymers make a significant percentage of the mixed berry diet. It is not known whether the dose of anthocyanins provided were too low to elicit any significant effect or whether there is a competition in the absorption of the different types of anthocyanins when presented as a mixture of berries. These results underscore the complexity of interactions between bioactive components present in berries.

Although it is clear that E2 treatment induces both 8-oxodG and enhances uncharacterized polar DNA adducts in vivo, the exact mechanism for this is unclear. The effect of E2 treatment on the expression of various CYP450 enzymes involved in its metabolism has been studied. Diethylstilbestrol, an E2 analogue, can induce CYP1A2 levels in mice liver (38). E2 itself has been shown to increase CYP1A2 expression in hamster liver (39). Further, CYP3A4 expression is influenced by estrogen receptor (40), and it is known that E2 induces the expression of several P450s involved in its metabolism (39). Thus, E2 treatment may induce an environment conducive for the production of its carcinogenic metabolites in the liver.

The possible mechanisms by which bioactive berry components may reduce DNA damage are summarized in Fig. 5. Partial evidence to support this scheme is available from previously published data. EA reduces the total hepatic P450 content and induces glutathione S-transferase activity in rat liver microsomes and inhibits CYP450 activity in vitro (23,41). Gallic acid and kaempferol, polyphenols found in berries, are known to inhibit CYP3A4 activity (25,42,43). The in vitro results from this study as well as our previous reports have shown that berry phytochemicals can actively inhibit DNA damage (24). We showed that berries and EA increase the expression of hepatic DNA repair enzymes (24). Moreover, data published by Xue and coworkers (44) also suggests that the inhibition of benzo-[α]pyrene-induced cell transformation by berry extracts, and EA may involve alteration of phase 1 metabolism as well as increase in DNA repair (44). Although, the protective effects of dietary berries and EA on hepatic DNA damage can be explained using unrelated accounts of their effect in various systems, it remains to be seen whether mechanisms postulated are indeed responsible for the reduction of both E2-induced hepatic DNA damage as well as mammary tumorigenesis in ACI rats.

Initial, unpublished data from our laboratory showed that dietary berries and EA are capable of countering the E2-induced levels of CYP1A1 mRNA in the ACI rat mammary (Aiyer and Gupta, unpublished results). It remains to be seen whether the bioactive berry components have similar effects on the hepatic CYP450s as well.

In conclusion, this report shows that berries and EA, 1 of the bioactive berry components, can effectively inhibit oxidative DNA damage caused by E2 and its metabolite 4E2, both in vivo and in vitro. Further studies are required to elucidate the exact mechanisms by which they produce these effects.

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