Carcinogenesis vol.30 no.7 pp.1202–1208, 2009
doi:10.1093/carcin/bgp093
Advance Access publication April 30, 2009

Vitamin C and α-naphthoflavone prevent estrogen-induced mammary tumors and decrease oxidative stress in female ACI rats

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The mechanisms underlying the pathogenesis of estrogen-induced breast carcinogenesis remain unclear. The present study investigated the roles of estrogen metabolism and oxidative stress in estrogen-mediated mammary carcinogenesis *in vivo*. Female August Copenhagen Irish (ACI) rats were treated with 17β-estradiol (E_2), the antioxidant vitamin C, the estrogen metabolic inhibitor α-naphthoflavone (ANF), or cotreated with E_2 + vitamin C or E_2 + ANF for up to 8 months. E_2 (3 mg) was administered as an subcutaneous implant, ANF was given via diet (0.2%) and vitamin C (1%) was added to drinking water. At necropsy, breast tumor incidence in the E_2, E_2 + vitamin C and E_2 + ANF groups was 82, 29 and 0%, respectively. Vitamin C and ANF attenuated E_2-induced alterations in oxidative stress markers in breast tissue, including 8-iso-prostane $F_{2\alpha}$ formation and changes in the activities of antioxidant enzymes superoxide dismutase and glutathione peroxidase. Quantification of 2-hydroxyestradiol (2-OHE_2) and 4-hydroxyestradiol (4-OHE_2) formation in breast tissue confirmed that ANF inhibited 4-hydroxylation of E_2 and decreased formation of the highly carcinogenic 4-OHE_2. These results demonstrate that antioxidant vitamin C reduces the incidence of estrogen-induced mammary tumors, increases tumor latency and decreases oxidative stress *in vivo*. Further, our data indicate that ANF completely abrogates breast cancer development in ACI rats. The present study is the first to demonstrate the inhibition of breast carcinogenesis by antioxidant vitamin C or the estrogen metabolic inhibitor ANF in an animal model of estrogen-induced mammary carcinogenesis. Taken together, these results suggest that E_2 metabolism and oxidant stress are critically involved in estrogen-induced breast carcinogenesis.

Introduction

Sex hormones have been implicated in the development of a number of human cancers, and recent data indicate that, in the USA, neoplasia of hormone-responsive tissues accounts for >35% of newly diagnosed cancers in men and >40% of newly diagnosed cancers in women (1). The importance of estrogens in the etiology of breast cancer is widely recognized, and the US government has added steroidal estrogens to the list of known human carcinogens (2–4). In general, elevated lifetime estrogen exposure is considered a major risk factor for breast cancer (5). While a growing body of clinical and epidemiological literature supports a role for estrogen in breast carcinogenesis, the exact mechanisms underlying the initiation and progression of estrogen-related cancers remain elusive (5).

Estrogens exert their carcinogenic effects via estrogen receptor (ER)-dependent mitogenic effects and ER-independent mechanisms (6,7). The ER-independent pathway of estrogen-induced breast carcinogenesis involves metabolic activation of endogenous estrogens by cytochrome P450 enzymes to generate highly reactive genotoxic metabolites (6). Cytochrome P450 1A1 (Cyp1A1) and Cytochrome P450 1B1 (Cyp1B1) are the primary enzymes responsible for the metabolism of 17β-estradiol (E_2) into the catechol estrogens 2-hydroxyestradiol (2-OHE_2) and 4-hydroxyestradiol (4-OHE_2), respectively (8). Tumorigenic estrogen metabolites such as 4-OHE_2 undergo oxidative metabolism to generate electrophilic quinones, which readily react with DNA to produce depurinating adducts and mutagenic reactive oxygen species (8–10). DNA adducts produced by the quinone forms of catechol estrogens have been detected in various tissues vulnerable to estrogen-induced carcinogenesis (11,12).

In this study, the antioxidant vitamin C and the E_2 metabolic inhibitor α-naphthoflavone (ANF) were used to investigate whether reducing oxidative stress and minimizing the metabolism of E_2 to catechol estrogens would reduce estrogen-induced tumor development *in vivo*. The August Copenhagen Irish (ACI) rat model of estrogen-induced breast cancer was used for the current study, as female ACI rats are uniquely sensitive to estrogen-induced breast carcinogenesis and develop tumors that are estrogen dependent, aneuploid and exhibit genomic instability (13–18). Female ACI rats were treated with vitamin C, ANF, E_2 and combinations consisting of either E_2 + vitamin C or E_2 + ANF for 7, 15, 120 and 240 days. Animals were examined daily for palpable tumors, and at the end of the experiments, animals were euthanized so that various tissues, including breast and liver, could be examined for both histopathological changes as well as alterations in the levels of oxidative stress markers.

Abbreviations: ACI, August Copenhagen Irish; ANF, α-naphthoflavone; CAT, catalase; E_2, 17β-estradiol; ER, estrogen receptor; GPx, glutathione peroxidase; 8-iso$PGF_{2\alpha}$, 8-iso-prostane $F_{2\alpha}$; 2-OHE_2, 2-hydroxyestradiol; 4-OHE_2, 4-hydroxyestradiol; SOD, superoxide dismutase.

Materials and methods

Chemicals

E_2, vitamin C, ANF, 2-OHE_2, 4-OHE_2 and cholesterol were purchased from Sigma–Aldrich (St Louis, MO). A total amount of 250 μCi [6,7-^{3}H(N)]-E_2 [specific activity 40–60 Ci (1.48–2.22 TBq)/mmol] was obtained from NEN Radiochemicals (Perkin Elmer, Waltham, MA).

Tumor development, estrogen, vitamin C and ANF treatment and histopathologic analysis

Female ACI rats (Harlan Sprague Dawley, Indianapolis, IN) were obtained at 4 weeks of age and housed in the Columbia University animal facility under controlled temperature, humidity and lighting conditions. Animals were fed AIN76A phytoestrogen-free diets (Dyets, Bethlehem, PA) and water was given *ad libitum*. After a one-week acclimatization period, rats were randomly distributed into six treatment groups. The experimental groups were treated with E_2, E_2 + vitamin C or E_2 + ANF. The respective controls groups were treated with cholesterol, cholesterol + vitamin C or cholesterol + ANF. Vitamin C (1%) was administered in drinking water and ANF was present in the diet (0.2%). Rats in the E_2, E_2 + vitamin C and E_2 + ANF treatment groups were implanted subcutaneously with E_2 pellets (3 mg E_2 + 17 mg cholesterol). Rats in the cholesterol, cholesterol + vitamin C and cholesterol + ANF control groups were implanted with pellets containing 17 mg cholesterol only. E_2 and cholesterol pellets were prepared using a pellet press as described previously (7,19–21). Vitamin C or ANF treatment began 7 days prior to pellet implantation. Each of the six treatment groups (cholesterol, cholesterol + vitamin C, cholesterol + ANF, E_2, E_2 + vitamin C and E_2 + ANF) was divided into four subgroups, containing at least 10 rats each, which underwent their respective treatments for 7, 15, 120 or 240 days. At the end of each of these time periods, animals were anesthetized using isoflurane and euthanized. Mammary (both tumor and normal), uterus, ovary, lung, brain, kidney and liver tissues were removed and snap frozen in liquid nitrogen for future analyses. Frozen tissues were stored at −70°C. A portion of the excised mammary and other tissues was stored in 10% buffered formalin for histopathological and immunohistochemical analyses. Tumor incidence and the number of tumor nodules per rat were counted at the time of dissection. The formalin-fixed tissue was embedded in paraffin, and sections of 4–5 μm thickness were cut. Paraffin-embedded sections of the mammary, liver, brain, uterus, kidney, lung and ovary were stained with hematoxylin

and eosin for histopathological evaluation by a pathologist. Histopathological analyses for tumor development and morphological changes were performed on mammary tissue from all rats from the experimental and control groups.

Determination of 8-iso-prostane $F_{2\alpha}$

Total 8-iso-Prostane $F_{2\alpha}$ (8-isoPGF$_{2\alpha}$) levels in the mammary, mammary tumor and liver tissue of female ACI rats were quantified using a direct 8-isoPGF$_{2\alpha}$ enzyme immunoassay kit obtained from Assay Designs (Ann Arbor, MI) according to the supplier's instructions as described previously (7,19). Breast and liver tissue homogenates were prepared for use in the 8-isoPGF$_{2\alpha}$ assay according to methods described previously (19). Data are expressed as mean 8-isoPGF$_{2\alpha}$ pg/mg protein ± SE of the mean. Fold changes were calculated by comparing 8-isoPGF$_{2\alpha}$ levels detected in the mammary or liver tissue of E_2, E_2 + vitamin C or E_2 + ANF-exposed animal tissues to levels in the mammary tissue of age-matched controls (i.e. the cholesterol, cholesterol + vitamin C or cholesterol + ANF groups).

Measurement of superoxide dismutase, catalase and glutathione peroxidase enzyme activity

Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities in the mammary, mammary tumor and liver tissues of ACI rats were measured using commercially-available kits from Cayman Chemical Company (Catalog Numbers 706002, 707002 and 703102, Ann Arbor, MI). Breast and liver tissue homogenates were prepared for use in the SOD, GPx and CAT assays according to methods described previously (19). SOD activity is reported as units/microgram protein. GPx activity is reported as nanomole/minute/milligram protein. CAT activity is reported as nanomole/minute/milligram protein. Fold changes in SOD, CAT and GPx activity were calculated by comparing the enzyme activities in the mammary, mammary tumor or liver tissue of E_2, E_2 + vitamin C and E_2 + ANF-treated rats to that of mammary or liver tissue from age-matched controls (i.e. E_2 versus cholesterol, vitamin C + E_2 versus vitamin C and E_2 + ANF versus ANF).

Quantification of 2- and 4-hydroxylation of E_2 in ACI rat breast tissue

This method was carried out according to previous descriptions (22,23). Breast tissue from rats in the cholesterol control, E_2, vitamin C, vitamin C + E_2, ANF and ANF + E_2 groups was homogenized in buffer containing 1.14% KCl/10 mM ethylenediaminetetraacetic acid at pH 7.5. Microsomes were isolated according to previously reported methods and stored in buffer containing 0.25 M sucrose/10 mM ethylenediaminetetraacetic acid, pH 7.5 (22–24). Protein concentrations in the microsome samples were determined by using the Pierce bicinchoninic acid Protein kit (Thermo Fisher, Waltham, MA). Radioactivity was assessed using a scintillation counter (Beckman Coulter, Fullterton, CA) and disintegrations per minute were counted and adjusted for microgram protein added to the assay. Data are expressed as a rate (picomolar catechol estrogen/minute/microgram).

Statistical analyses

All data were analyzed using Sigma Plot 8.0 (Systat Software, San Jose, CA). Fisher's exact test was used to compare tumor incidence between two treatment groups or between a treatment group and a specific control group. The average number of tumor nodules per tumor-bearing animal was calculated by dividing the sum of the tumor nodules in all tumor-bearing animals by the total number of tumor-bearing animals. The average number of tumor nodules per rat is expressed as the mean ± SE. After calculating Kaplan–Meier survival curves of tumor occurrence for latency, we used log-rank test to detect differences in the survival curves between two treatment groups. The two sample *t*-test was used to detect group differences in quantitative variables, including tumor multiplicity (defined as number of tumor nodules per tumor-bearing rat) and specific fold changes in 8-isoPGF$_{2\alpha}$ levels as well as in SOD, GPx and CAT activities.

Results

ANF and vitamin C significantly decrease the development of E_2-induced mammary tumors

Mammary tissue from all animals in the control groups (cholesterol, cholesterol + vitamin C and cholesterol + ANF) displayed normal lobular architecture, consisting of ducts surrounded by small lobules (Figure 1). No morphological differences were detected between mammary tissues from any of the control groups (Figure 1). Analysis of mammary tissue from all rats in the E_2-treated experimental groups (i.e. E_2, vitamin C + E_2 and ANF + E_2-treated animals) revealed hyperplastic lobular units that expanded into the stromal fat (Figure 1). Lobular hyperplasia and compression of stromal fat progressively increased with duration of E_2 exposure (data not shown). Mammary tissue from rats treated with E_2, E_2 + vitamin C or E_2 + ANF were not morphologically different from one another, indicating that neither vitamin C nor ANF interfered with the proliferative response of the mammary gland to E_2 (Figure 1). No mammary tumors were observed in control rats (Table I). The incidence of mammary tumors in rats treated with E_2 was equal to 82% following 240 days (Table I). Strikingly, cotreatment of rats with ANF + E_2 completely abrogated the development of mammary tumors (Table I). Mammary tumor incidence in rats in the E_2 + vitamin C group was equal to 29% after 240 days treatment (Table I). The first palpable breast tumor appeared after 128 days in the E_2-treatment group, whereas in the E_2 + vitamin C group, the first palpable tumor did not appear until day 183 (Table I,

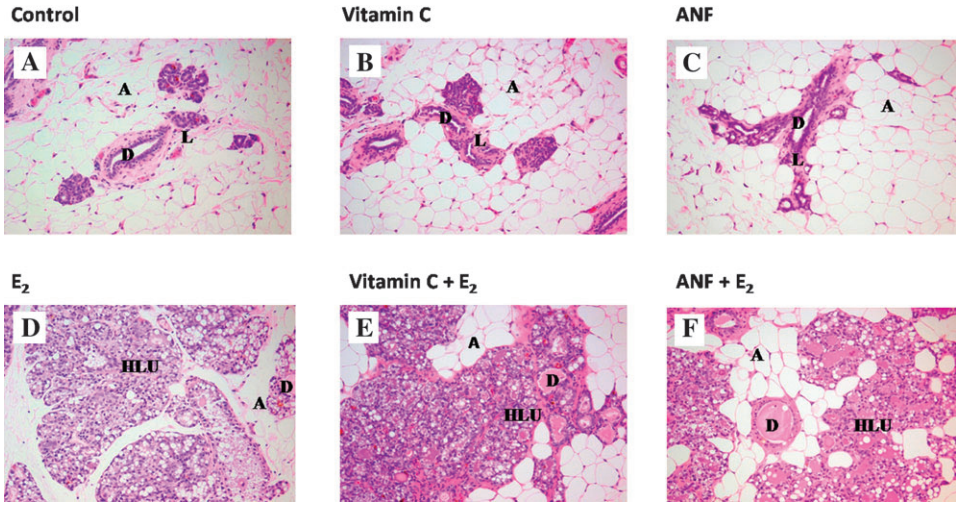

Fig. 1. Mammary tissue from the E_2, E_2 + vitamin C, E_2 + ANF and their respective control groups (i.e. cholesterol, cholesterol + vitamin C and cholesterol + ANF) after 240 days. Female ACI rats were treated with E_2, E_2 + vitamin C or E_2 + ANF for 240 days. Animals in the E_2, E_2 + vitamin C and E_2 + ANF groups were implanted with E_2 pellets (subcutaneous, 3 mg E_2 + 17 mg cholesterol) for 240 days. Control rats were implanted with pellets containing 17 mg cholesterol only. Vitamin C-treated rats received vitamin C (1%) in drinking water and ANF-treated rats were given ANF via diet (0.2% in food). (**A**–**C**) The mammary glands of ACI rats exposed to cholesterol, cholesterol + vitamin C or cholesterol + ANF show normal lobular architecture (L) with branched ducts (D) and normal distribution of fat/adipose tissue (A) (all panels ×100). (**D**–**F**) Mammary glands from rats in the E_2, vitamin C + E_2 and ANF + E_2 groups show increased proliferation with dilated ducts containing inspissated secretions (D) and increased proliferation and expansion of terminal lobular units (HLU) accompanied by compression of and expansion into the surrounding fat/adipose tissue (A) (all panels ×100).

Table I. Tumor incidence and multiplicity by treatment group

Treatment	Tumor incidence	Tumor multiplicity	Day of first tumor appearance
Cholesterol	0 (0/10)	NA	NA
17β-Estradiol (E_2)	81.8% (9/11)	3.1 ± 0.7	128
Vitamin C	0 (0/17)	NA	NA
Vitamin C + E_2	29.4% (5/17)*	1.6 ± 0.4	183
α-naphthoflavone (ANF)	0 (0/18)	NA	NA
ANF + E_2	0 (0/17)*	NA	NA

NA, not applicable.
Column one lists the different treatments each group of animals received. The percentage of animals that developed tumors during the 240 day treatment period (tumor incidence) is listed in column two. The symbol indicates '*' indicates a P value <0.05 comparing tumor incidence between the E_2 treatment group and the E_2 + vitamin C and E_2 + ANF treatment groups. P-values were calculated using Fisher's exact test. The number of tumors per tumor-bearing rat (tumor multiplicity) is listed in column three. Column four lists the day of first tumor appearance for animals in each group.

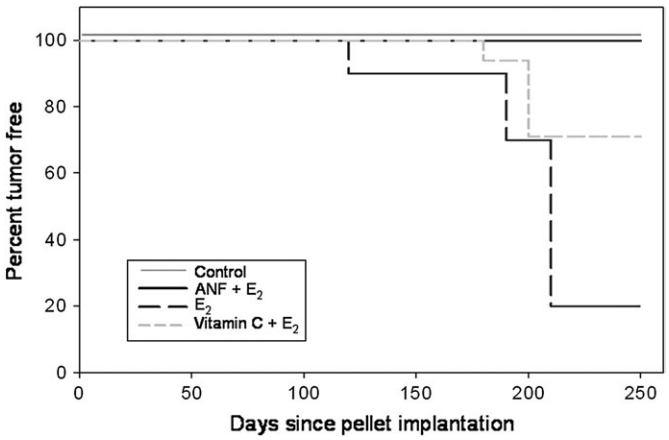

Fig. 2. Vitamin C and ANF prevent the development of E_2-induced breast tumors. Female ACI rats were treated with E_2, E_2 + vitamin C or E_2 + ANF as described in the Materials and Methods section. Kaplan–Meier survival curves for tumor occurrence were plotted for each treatment group, and the log-rank test was used to detect differences in the tumor latency curves between groups. Animals in the control groups (cholesterol, cholesterol + vitamin C and cholesterol + ANF) did not develop any tumors and are represented by the same line on the graph (Control). Tumor latency was significantly longer in the vitamin C + E_2 group compared with the E_2 group ($P = 0.01$).

Figure 2). Indeed, according to Kaplan–Meier survival curve analysis, average tumor latency was significantly longer for animals in the vitamin C + E_2 group relative to those in the E_2 group (Figure 2). Tumor multiplicity was not significantly reduced by vitamin C; however, the data suggest a trend toward decreased tumor multiplicity in the vitamin C + E_2 group relative to the E_2 group (Table I). Histopathological examination revealed no differences in tumor type or morphology between tumors collected from animals in the E_2 and E_2 + vitamin C groups. Mammary tumors from both groups were classified as adenocarcinomas (data not shown). Breast tumors from E_2-treated rats showed evidence of invasion, whereas tumors from animals in the E_2 + vitamin C group were mostly encapsulated (data not shown).

Quantification of 2- and 4-hydroxylation of E_2 in ACI rat breast

The 2- and 4-hydroxylase activity assay was used in order to examine estrogen metabolism in the breast tissue of rats treated with cholesterol, E_2, vitamin C and ANF groups. In rat breast, 4-hydroxylation of E_2 represents the major pathway of estrogen metabolism, whereas 2-hydroxylation makes up a relatively minor fraction of reactions. This assay was performed in order to confirm that ANF treatment inhibited the formation of 4-OHE_2, as has been reported previously (25–28). Microsomes from rats in the cholesterol, cholesterol + vitamin C and cholesterol + ANF groups catalyzed the formation of very little 2- and 4-OHE_2 (Figure 3). No significant differences were observed between the formation of 2- or 4-OHE_2 in the cholesterol, cholesterol + vitamin C or cholesterol + ANF groups, vitamin C or ANF groups. Similarly, microsomes from rats in the E_2, vitamin C + E_2 and ANF + E_2 groups catalyzed very little 2-hydroxylation of E_2, and 2-OHE_2 generation in these groups was not significantly different from formation observed in control, vitamin C or ANF-treated animals. However, 4-OHE_2 formation by microsomes from rats in the E_2 and vitamin C + E_2 groups was greatly increased compared with rats from the cholesterol and cholesterol + vitamin C groups, respectively (Figure 3). Notably, microsomes from rats in the ANF + E_2 group catalyzed very little 4-hydroxylation of E_2 (Figure 3). 4-OHE_2 formation in the ANF + E_2 group was comparable with 4-OHE_2 formation observed in cholesterol- and cholesterol + ANF- treated control animals and was significantly lower than 4-OHE_2 generation observed in microsomes from E_2 and vitamin C + E_2-treated rats ($P < 0.01$). These results confirm that ANF treatment prevents 4-hydroxlation of E_2 in ACI rat breast tissue.

ANF and vitamin C treatment reduce E_2-associated 8-isoPGF$_{2\alpha}$ formation

8-isoPGF$_{2\alpha}$, a known marker of lipid peroxidation and oxidative stress, was used to assess oxidative stress in mammary, mammary tumor and liver tissues from ACI rats (29). No significant differences in liver 8-isoPGF$_{2\alpha}$ levels were detected between control animals and those in the E_2, E_2 + vitamin C or E_2 + ANF groups after 240 days treatment (data not shown). As reported recently, mammary tissue 8-isoPGF$_{2\alpha}$ levels were increased in a time-dependent manner following exposure to E_2 (19). Vitamin C and ANF suppressed E_2-induced elevations in mammary 8-isoPGF$_{2\alpha}$ formation. At the 240 day time point, mammary tissue from rats in the vitamin C + E_2 or ANF + E_2 groups displayed significantly smaller fold increases in 8-isoPGF$_{2\alpha}$ levels relative to rats in the 240 day E_2 treatment group (Figure 4). As reported recently, mammary tissue 8-isoPGF$_{2\alpha}$ levels in rats treated with E_2 for 240 days were ~5-fold higher than in age-matched cholesterol-treated controls (19). In contrast, mammary 8-isoPGF$_{2\alpha}$ levels were elevated 2.5- and 2.4-fold after 240 days treatment with vitamin C + E_2 and ANF + E_2, respectively (Figure 4).

ANF and vitamin C prevent E_2-induced alterations in the activities of antioxidant enzymes

The activities of the antioxidant enzymes GPx, CAT and SOD were quantified in mammary, mammary tumor and liver tissues from rats in all of the experimental and control groups. No significant alterations in GPx or SOD activity were detected in mammary tissue from rats treated with vitamin C + E_2 or ANF + E_2 for 7, 15, 120 or 240 days relative to their respective age-matched controls (Figure 5). Furthermore, mammary tumors from animals in the E_2 + vitamin C group did not display changes in SOD or GPx activity relative to control breast tissue (Figure 5). The lack of change in SOD or GPx activity in animals from the vitamin C + E_2 or ANF + E_2 groups is in contrast with increases in SOD and GPx activity observed in mammary and mammary tumor tissue from animals treated with E_2 alone (Figure 5). No alterations in CAT activity were detected in mammary tissue from animals in the E_2 + ANF or E_2 + vitamin C groups (data not shown). No changes in SOD, GPx or CAT enzyme activity were observed between the liver tissues of control and E_2, vitamin C + E_2 or ANF + E_2-treated rats (data not shown).

Discussion

Both epidemiological and experimental evidence implicate estrogens in the pathogenesis of breast cancer. Despite extensive research, the

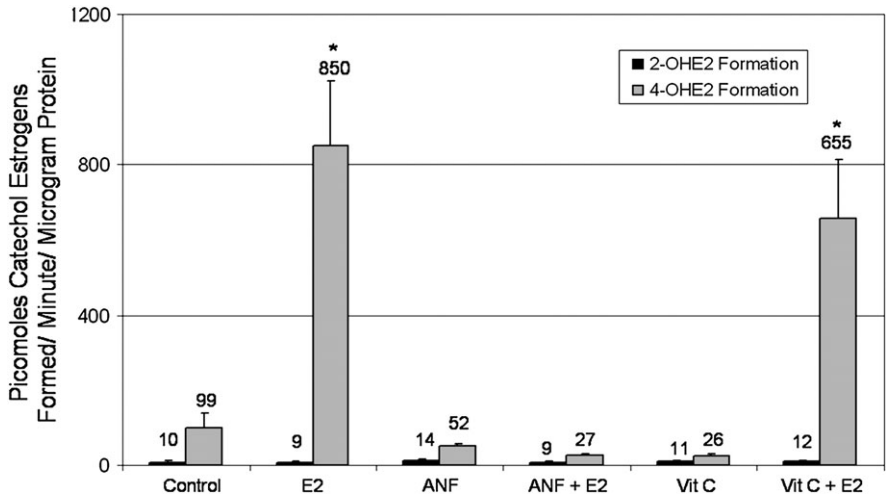

Fig. 3. Quantification of 2- and 4-hydroxylation of E_2 in rat breasts. The 2- and 4-hydroxylase activity assay was used in order to examine estrogen metabolism in the breast tissue of rats from the cholesterol, cholesterol + vitamin C, cholesterol + ANF, E_2, vitamin C + E_2 and ANF + E_2 groups. Radioactive ^{3}H-E_2 was used to trace the formation of catechol estrogens by rat breast microsomes. The 2- and 4-OHE_2 generated during the reaction were separated from one another by using a thin layer chromatography method, and the amounts of each radioactive catechol estrogen were quantified by using a scintillation counter and corrected for the amount of microsomal protein added to the reactions. Data are expressed as picomoles catechol estrogen formed/minute/μg protein ± standard error. The symbol '*' indicates a P value <0.05 compared with control values. The absolute values are listed above each bar. No differences in 2-hydroxyestradiol (2-OHE_2) formation were detected between any of the treatment groups. 4-OHE_2 generation was significantly higher than control levels in the E_2 and vitamin C + E_2 groups. The presence of ANF inhibits E_2-induced increases in 4-hydroxylation in rat breast.

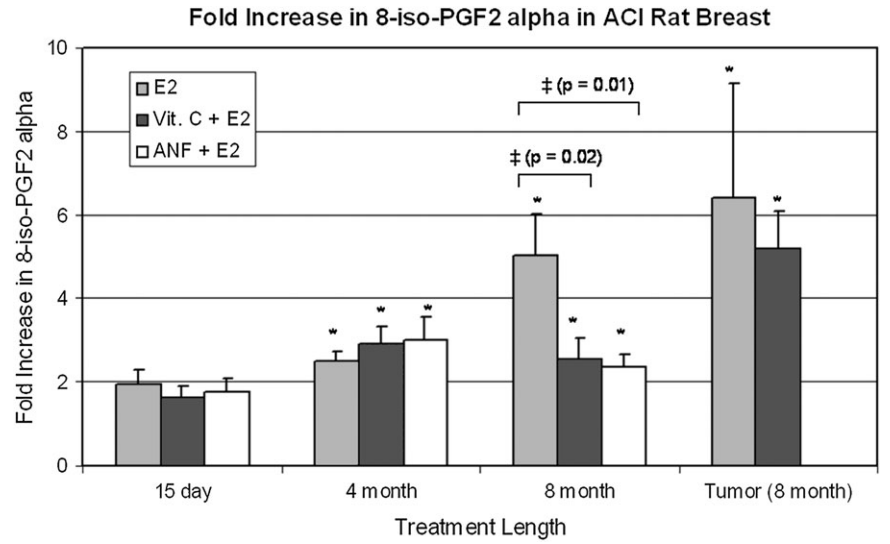

Fig. 4. Vitamin C and ANF attenuate E_2-associated increases in 8-isoPGF$_{2\alpha}$. Female ACI rats were treated with cholesterol, cholesterol + vitamin C, cholesterol + ANF, E_2, E_2 + vitamin C or E_2 + ANF as described in the Materials and Methods section. 8-isoPGF$_{2\alpha}$ levels were measured in mammary tissue from animals in each of these groups. 8-isoPGF$_{2\alpha}$ levels were assessed in mammary tumor tissue as well. Fold changes were calculated for E_2-treated animals relative to age-matched cholesterol-treated controls. Fold changes were calculated for E_2 + vitamin C-treated animals relative to age-matched vitamin C-treated controls. Fold changes were calculated for E_2 + ANF-treated animals relative to age-matched ANF-treated controls. Fold change data for tumor tissue were determined by comparing 8-isoPGF$_{2\alpha}$ levels in tumor tissue with levels detected in non-tumor mammary tissue from age-matched control rats. These data are reported as an average of values obtained for at least seven different animals ± standard error. The symbol '*' indicates a P value <0.05 relative to each group's respective controls. The symbol '‡' indicates a P value <0.05 comparing E_2 and E_2 + vitamin C groups or E_2 and E_2 + ANF groups.

mechanisms by which estrogens exert their carcinogenic effects remain elusive (5,30). Current data suggest that tumor induction by E_2 depends on E_2 metabolism and subsequent oxidative stress (7,27,31–33). The present study was designed to evaluate the importance of oxidative stress and estrogen metabolism in breast carcinogenesis using an *in vivo* model. Female ACI rats were treated with E_2 with or without simultaneous treatment with vitamin C or ANF. Vitamin C, an antioxidant, was used to reduce the burden of oxidative stress during E_2 treatment. ANF, a Cyp inhibitor, has previously been shown to inhibit estrogen metabolism and decrease the formation of catechol estrogens (25,34). In this study, ANF was used to minimize E_2 metabolism and reduce the production of catechol estrogens. Catechol estrogens, such as 4-OHE_2, can form depurinating DNA adducts and/or produce oxidative stress via metabolic redox cycling, a pathway that is proposed to play an important role in hormonal carcinogenesis (7,30).

Demonstrating the critical importance of estrogen metabolism and oxidative stress in breast carcinogenesis, ANF treatment completely abrogated the development of mammary tumors, and vitamin C treatment significantly reduced tumor incidence and significantly increased tumor latency in female ACI rats (Figure 2, Table I). Moreover, the

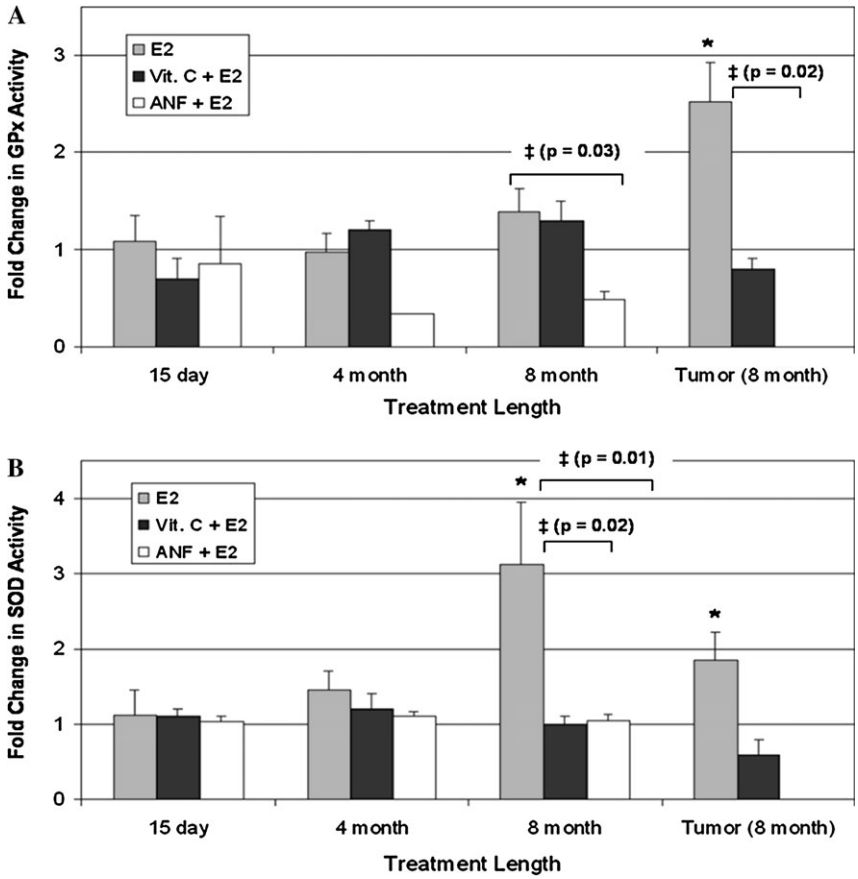

Fig. 5. Vitamin C and ANF decrease E_2-induced changes in antioxidant enzyme activities. Female ACI rats were treated with cholesterol, E_2, cholesterol + vitamin C, E_2 + vitamin C, ANF or E_2 + ANF as described in the Materials and Methods section. The activity levels of SOD and GPx were measured in mammary tissue and mammary tumor tissue from animals in each of the treatment groups. Fold changes in enzyme activity were calculated for animals in the E_2, E_2 + vitamin C and E_2 + ANF groups relative to enzyme activity levels in age-matched control animals. Fold change data for tumor tissue were determined by comparing enzyme activity levels in tumor tissue to levels observed in non-tumor mammary tissue from age-matched controls. These data are reported as an average of values obtained for at least seven different animals ± standard error. (**A**) Fold changes in GPx activity in mammary tissue from rats treated with E_2, E_2 + vitamin C and E_2 + ANF. (**B**) Fold changes in SOD activity in mammary tissue from rats treated with E_2, E_2 + vitamin C and E_2 + ANF. The symbol '*' indicates a *P* value <0.05 relative to each group's respective controls. The symbol '‡' indicates a *P* value <0.05 comparing the E_2 + vitamin C and E_2 + ANF groups to the E_2 group.

results of our histopathological analyses showed that vitamin C and ANF had no effect on proliferation of mammary tissue in ACI rats, such that mammary tissues from animals in the E_2, vitamin C + E_2 and ANF + E_2 groups were not markedly different from one another (Figure 1). The proliferative response of the breast to E_2 remains intact in animals from each of the treatment groups, and the decrease in E_2 metabolism and oxidative stress in the E_2 + ANF and E_2 + vitamin C groups is probably responsible for the dramatic reduction in breast cancer observed in these groups. ANF has previously been shown to inhibit Cyp activity and E_2 metabolism (25,26,35).

In contrast to the distinct effects of E_2 treatment on mammary tissue, no significant morphological changes were observed in kidneys, uteri, lungs or brains from rats in the E_2, vitamin C + E_2 or ANF + E_2 groups relative to tissues collected from age-matched controls (data not shown). The ANF dose (0.2% wt/wt in diet) chosen for use in this study effectively prevented the development of estrogen-dependent renal tumors in Syrian hamsters without dramatically elevating liver tumor incidence; however, higher ANF doses (0.4% wt/wt in diet) augmented development of liver tumors in hamsters (27,28).

ANF and vitamin C reduced E_2-associated elevations in oxidative stress in ACI rats. Mammary tissue from rats cotreated with either vitamin C + E_2 or ANF + E_2 for 240 days displayed significantly smaller fold increases in 8-isoPGF$_{2\alpha}$ levels, an established marker of oxidative stress *in vitro* and *in vivo*, relative to rats treated with E_2 only (Figure 4) (36). Similarly, ANF and vitamin C attenuated E_2-induced increases in the activities of the antioxidant enzymes SOD and GPx (Figure 5). The implication of these findings is that animals exposed to E_2 undergo increases in oxidative stress and compensate for this stress by enhancing the activities of detoxifying enzymes, such as SOD and GPx. The observation that animals treated simultaneously with E_2 and either vitamin C or ANF do not display compensatory increases in the activities of antioxidant enzymes and have significantly smaller increases in 8-isoPGF$_{2\alpha}$ than do animals treated with E_2 only suggests that the vitamin C and ANF suppress E_2-induced oxidative stress and at least partially block the receptor-independent/genotoxic metabolite pathway of tumor induction by E_2. Detection of increased SOD activity in breast tissue from rats treated with E_2 for 240 days without corresponding increases in GPx or CAT activity suggests that H_2O_2 may accumulate in the breast tissue of E_2-treated rats. Increases in SOD activity were not detected in breast tissue from animals treated with E_2 + vitamin C or E_2 + ANF, indicating that accumulation of H_2O_2 may not occur in these animals. Furthermore, no changes in 8-isoPGF$_{2\alpha}$ levels or antioxidant enzyme activities were found in liver tissue from rats treated with E_2 or E_2 + vitamin C for any of the time points (data not shown), suggesting that oxidative changes may only occur in estrogen-target tissues.

The present study is the first report demonstrating the inhibition of estrogen-induced breast carcinogenesis by antioxidant vitamin C or the estrogen metabolic inhibitor ANF in an animal model of estrogen-induced breast cancer. The results of the present investigation are in agreement with previous reports that ANF and vitamin C reduce

estrogen-induced renal carcinogenesis in hamsters (27,28,37–39). Presumably, the protective effects of vitamin C and ANF stem from their abilities to prevent oxidation of estrogens and reduce the generation of carcinogenic metabolites. The carcinogenicity of 4-OHE_2, the principal genotoxic metabolite of E_2, has been demonstrated in the hamster renal tumor model as well as in the CD-1 mouse uterine tumor model (32,33). In human breast tumor biopsies, 4-hydroxlation of E_2 was elevated relative to measurements in normal human breast tissue (40,41). Surprisingly, a recent study showed that 4-OHE_2 exposure was not sufficient to produce mammary tumors in female ACI rats (42). While this result seems paradoxical, it is possible that exposure to exogenous 4-OHE_2 does not produce target organ concentrations great enough to induce cancer development, as 4-OHE_2 is more water soluble than E_2 and is cleared more quickly. The authors suggest that it may be critical for 4-OHE_2 to be formed in or near the target tissue in order to reach sufficient levels and effectively initiate tumorigenesis (42).

Further illustrating the importance of oxidative stress in initiating breast carcinogenesis, a number of epidemiologic investigations have revealed that vitamin C in the form of fruits and vegetables may lower breast cancer risk. A meta-analysis of 12 case–control studies related to diet and breast cancer demonstrated that vitamin C intake via fruit and vegetable consumption reduces breast cancer risk (relative risk for highest versus lowest quintile of consumer, 0.69; $P < 0.0001$) (43). Unfortunately, in prospective studies, the purported inverse association between breast cancer risks and vitamin C intake did not materialize (44–48). However, upon examination of specific risk categories, two well-designed prospective trials detected significant decreases in breast cancer among women in the quintile of highest vitamin C-rich food consumption compared with women in the lowest quintile. Similarly, the Nurses' Health Study has shown that premenopausal women with a family history of breast cancer who consume an average of 205 mg/day of vitamin C from food have a 63% lower risk of breast cancer than those who consumed an average of 70 mg/day ($P = 0.002$) (49). The Swedish Mammography Cohort, a population-based mammography screening program, reported a 39% lower risk of breast cancer among overweight (BMI >25 kg/m^2) women who consumed an average of 110 mg/day of vitamin C, relative to overweight women who consumed just 31 mg/day ($P = 0.004$) (50).

Taken together, the results of the present study demonstrate that while inhibition of oxidative stress significantly reduces breast tumorigenesis, inhibition of estrogen metabolism, a prerequisite for the generation of reactive oxygen species and cellular damage by genotoxic metabolites, completely abrogates breast tumor development in this animal model. Thus, estrogen metabolism-mediated oxidative stress plays an important role in the process of E_2-induced breast carcinogenesis. Use of vitamin C and ANF to minimize the contribution of the ER-independent pathway of breast cancer induction significantly reduced mammary tumor development in the ACI rat model of estrogen-dependent breast carcinogenesis. These data strongly suggest that E_2 metabolism and oxidative stress are critical factors in the development of mammary tumors following E_2 exposure, as even partially decreasing the action of this receptor-independent pathway significantly impairs tumor development.

Funding

National Institutes of Health (ES009089, CA 109551 to H.K.B.).

Acknowledgements

Conflict of Interest Statement: None declared.

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Received February 27, 2009; revised April 9, 2009; accepted April 10, 2009