Review Article

17β-Estradiol epoxidation as the molecular basis for breast cancer initiation and prevention

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Epidemiological and animal studies have indicated that 17β-estradiol (E2) is involved in breast cancer; however, the mechanism is unclear. We found that E2 could be activated by epoxidation, resulting in its ability to inhibit nuclear DNA-dependent RNA synthesis, and to bind DNA, forming DNA adducts both in vitro and in vivo. Because epoxidation is required for the activation of many chemical carcinogens, including benzo(a)pyrene, 7,12-dimethylbenz(a)anthracene and aflatoxins, we proposed previously that E2 epoxidation is the underlying mechanism for the initiation of breast cancer. The first part of this review is to present the experimental evidence obtained from this laboratory in support of this hypothesis. Based on these newly discovered insights on E2 epoxidation and its initiation role in breast cancer carcinogenesis, a method to screen chemopreventive agents against breast cancer has been developed. This constitutes the second part of the review. Two examples will be used to illustrate the utility of this screening technique. The effect of fat on breast cancer has been a longstanding but unresolved issue. Epidemiological studies provide conflicting results regarding the association of dietary fat and breast cancer. Because vegetable oils contain various amounts of mono- and polyunsaturated fatty acids, they are potential antioxidants. Data are presented to show that commercial vegetable oils, independent of their mono- or polyunsaturated fatty acid content, are all able to prevent the formation of E2 epoxide, as measured by the loss of the ability of E2 to inhibit nuclear RNA synthesis in vitro. Tamoxifen (TAM), an anti-estrogen used for breast cancer treatment, has recently been found to have a strong breast cancer preventive effect. The mechanism for this is unknown. Using the same screening technique, we found that when incubated together with E2 for epoxidation, TAM was able to prevent the formation of E2 epoxide, as evidenced by both the loss of the ability of E2 to inhibit nuclear RNA synthesis and the reduced binding of [3H]-labelled E2 to nuclear DNA in a dose-dependent manner. These experimental results suggest that the breast cancer preventive effect of TAM is to prevent the formation of E2 epoxide through a competitive epoxidation mechanism with E2.

Key words: 17β-estradiol, 17β-estradiol epoxide, breast cancer, chemopreventive agent screening, DNA adduct, RNA synthesis, tamoxifen, vegetable oil.

Introduction

Breast cancer is the most common form of cancer among US women, with an estimated 183,000 new cases each year, and is the second leading cause of cancer deaths estimated at 41,000 per year.1 Animal and epidemiological studies have indicated that oestrogens are involved in uterine2,3 and breast4-6 cancers. However, their mechanisms are still not well understood.7 Basically, there are two ways to fight and win the war on cancer. One is to find a means of preventing the disease and the other is to find a way to successfully treat it. Studies indicate that as many as 80% of all cancers are related to environmental or external factors and are therefore, in theory, preventable.8,9 However, at present, because our understanding of cancer is still very limited, we cannot prevent cancers from occurring. In addition, there are social, political, economical and personal factors that may prevent the application of our knowledge in cancer prevention. An additional complication in breast cancer prevention is that 17β-estradiol (E2) and oestrone (E1) are endogenous hormones. Furthermore, oestrogens (either natural or synthetic) are widely used in a variety of clinical conditions from oestrogen replacement therapy to cancer treatment, irrespective of the fact that they are known to be carcinogens.2-6 Earlier studies have clearly demonstrated that the synthetic oestrogen diethylstilbestrol (DES), used with the goal of stabilising pregnancies, has been associated with an increased risk of breast cancer in those women who take it,10 and of vaginal adenocarcinoma in their daughters.11 Results from oestrogen replacement studies have indicated that although exposure to exogenous oestrogen for less than two years does not increase the risk of breast cancer, extended periods of use lasting more than 10 years may increase the risk by 25-30%.12 Data from recent prospective case-control studies have clearly shown that there is a positive association between blood levels of E2 and E1 and the risk of breast cancer in postmenopausal women.4-6 Clearly, the basic understanding of the molecular...
mechanism of these carcinogens is fundamentally important for the proper design of breast cancer prevention strategies and treatment of the disease.

**17β-estradiol epoxidation as the underlying mechanism of breast cancer initiation**

Several years ago, we found that E1 and E2 could be activated by the epoxide-forming oxidant dimethyldioxirane (DMDO). This resulted in the inhibition of rat liver nuclear and nucleolar RNA synthesis *in vitro* (Fig. 1).13 Because epoxidation is required for the activation of many well-known chemical carcinogens (e.g., benzo(a)pyrene, 7,12-dimethylbenz(a)anthracene and aflatoxins),14–23 we proposed that oestrogen epoxidation is the underlying mechanism for the initiation of breast cancer (Fig. 2).13

Chemical carcinogenesis is a multistage process that includes initiation, promotion and progression.14–19 Initiation, the first critical and irreversible step in carcinogenesis, requires the covalent binding of a carcinogen to DNA.14–19 For this reason, one of the basic tests of our hypothesis was to determine whether E1 and E2 are able to bind to DNA after epoxide activation. In support of our hypothesis, we found that [3H]-labelled E1 and E2 are able to bind to DNA only after epoxide activation using several different DNA templates (Fig. 3).24,25 The covalent binding nature of E1 and E2 to DNA was further confirmed by [32P]-post-labelling analysis (Fig. 4).24–26

However, as these results were obtained mainly from *in vitro* experiments, it is important to show that oestrogen DNA adducts are also formed *in vivo*. A recent report of female ACI rats showed that when a continuous treatment of E2 was delivered through Silastic tubing implants containing 27.5 mg crystalline E2, 100% of the rats developed mammary tumours within a year.27 Using the same strain of

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**Figure 1.** Dose–response inhibition curves of (×–×) dimethyldioxirane (DMDO)-activated estrone (E1), (○–○) 17β-estradiol (E2), (□–□) diethylstilbestrol (DES) and (●–●) tamoxifen (TAM) on rat liver nuclear RNA synthesis *in vitro*. Values given are the mean of 2–4 separate experiments. (Reproduced, with permission, from reference 13.)

**Figure 2.** Epoxidation of estrogen as the basis for carcinogenesis.

**Figure 3.** Binding of [3H]-labelled 17β-estradiol (E2) epoxide to (○–○) calf thymus DNA, (●–●) poly(●(A-T)) and (×–×) poly(●(G-C)). Values given are the average of 2–3 separate experiments. (Reproduced, with permission, from reference 14.)

**Figure 4.** 32P-postlabelling maps of the 17β-estradiol (E2) and dimethyldioxirane (DMDO)-activated E2-treated calf thymus DNA. (a) Control group; (b) E2 epoxide group. (Reproduced, with permission, from reference 25.)
rats, we found that when the female ACI rats were given intramammillary injections of E₂ or DMDO-activated E₂ (i.e., E₂ epoxide), identical DNA adducts were formed in vivo, and the E₂ epoxide was at least 25,000 times more active than E₂ in the formation of DNA adducts in mammary glands (Figs 5,6). Therefore, these in vitro and in vivo experiments have provided critical evidence in support of our proposed hypothesis of oestrogen epoxidation and the initiation of oestrogen carcinogenesis.13

17β-estradiol epoxidation as a molecular basis for breast cancer prevention
It is clear that in order to properly prevent a disease, it is necessary to know the cause of the disease. In terms of breast cancer, our findings that E₂ could be activated by epoxidation13 and was consequently able to bind DNA, forming DNA adducts, in vitro and in vivo24–26,28–30 have provided a strong molecular basis for an initiatory role of E₂ in breast cancer aetiology.13 Based on this new insight, we

Figure 5. In vivo detection of 17β-estradiol (E₂)-DNA adducts in the mammary glands of female ACI rats after intramammillary injections of E₂ or E₂ epoxide. (a) Control group: only the solvent, 20% DMSO in corn oil, was injected. (b) E₂ group: single injection of 250 μg/mammary gland per day for three consecutive days. (c) E₂ epoxide group: single injection of 1 μg/mammary gland. (Reproduced, with permission, from reference 30.)

Figure 6. In vivo evidence for the formation of identical 17β-estradiol (E₂)-DNA adducts in the mammary glands of female ACI rats given intramammillary injections of E₂ or E₂ epoxide. The major DNA adducts, namely 1, 2 and 3, from both E₂ and E₂ epoxide groups were excised, eluted, concentrated and analysed by thin-layer chromatography under four different solvent systems. (a) 0.4 M Tris-HCl, 0.4 M H₃BO₃, 8 mM EDTA, 1.04 M NaCl, and 6.4 M urea (pH 8); (b) Isopropanol: 4 N NH₄OH (1:1, v/v); (c) 0.56 M LiCl, 0.24 M NaH₂PO₄, 0.4 M Tris-base, and 6.8 M urea (pH 4.5); (d) 0.64 M NaH₂PO₄, 0.4 M Tris-HCl and 6.8 M urea (pH 8.0). (i) 1, 2 and 3 are the three major DNA adducts from the E₂ group, as shown in Figure 5. (ii) 1, 2 and 3 are the three major DNA adducts from the E₂ epoxide group, as shown in Figure 5. (Reproduced, with permission, from reference 30.)
have developed a technique to screen potential chemopreventive agents at the initiation step of breast cancer carcinogenesis. This screening test determines whether a chemical agent is able to prevent the formation of £E2 epoxide (i.e., prevention at the initiation step), as measured by both the loss of the ability of £E2 to inhibit nuclear DNA-dependent RNA synthesis$^{13,24,25}$ and the ability of [H]-labelled £E2 to bind to nuclear DNA.$^{24-26,28-30}$ The following two examples are used to illustrate the utility of this screening technique.

1. Evidence for the potential of vegetable oils in breast cancer prevention

The effect of dietary fat on breast cancer has been a longstanding and unresolved issue.$^{31-33}$ Although it is a popular belief that monounsaturated fat (e.g., olive oil) protects and polyunsaturated fat (e.g., linoleic acid) promotes breast cancer carcinogenesis,$^{33-35}$ results from recent large-scale epidemiological studies have found no evidence that the intake of either total fat or specific subtypes of fat were associated with breast cancer risk.$^{36-38}$ There are at least two basic reasons why this issue has not been resolved for so long: (i) Epidemiological studies measuring dietary intake do not take into consideration other lifestyle risk factors (e.g., obesity, physical activity and other eating habits) that may contribute to the final outcome of the disease; (ii) Chemical carcinogenesis is a multistage process$^{14-19}$ and epidemiological studies are not able to differentiate the beneficial or harmful effects of dietary fat at a defined stage during the multistage process of carcinogenesis. Based on the above analyses, it is clear that in order to have a better understanding of the effect of dietary fat on breast cancer, it is necessary to dissect and study the effect of dietary fat at the individual steps of the multistage process of chemical carcinogenesis.

Because vegetable oils contain various amounts of mono- and polyunsaturated fatty acids, they are potential antioxidants. The results from our studies, as shown in Fig. 7, indicate that commercial vegetable oils, independent of their mono- or polyunsaturated fatty acid content, are all able to prevent the formation of £E2 epoxide, as measured by the loss of the ability of £E2 to inhibit nuclear RNA synthesis in vitro.

These are very dramatic findings. Basically, these results confirm our belief that vegetable oils are effective anti-oxidants and are able to prevent the formation of £E2 epoxide in vitro. However, because vegetable oils are heated in cooking (except when used in salad dressing), and because heating may cause oxidation of the unsaturated fatty acids in the vegetable oil, possibly causing them to lose their protective effect against £E2 epoxidation, it is important to know whether heating will abolish the protective effect of the vegetable oils. As indicated in Table 1, heating the vegetable oils at 200°C for 5 min did not reduce this preventive effect.

2. Prevention of £E2 epoxide formation through competitive epoxidation as the mechanism for tamoxifen in breast cancer prevention

Tamoxifen has been used for adjuvant therapy in breast cancer treatment since the early 1970s. Recent large clinical trials indicate that TAM is also an effective chemopreventive agent for breast cancer.$^{39}$ Because TAM is known to block the binding of £E2 to its receptor, this anti-estrogen action is believed to be the underlying mechanism for the efficacy of

Table 1. Preventive effect of vegetable oil on the inhibition of nuclear RNA synthesis by 17β-estradiol (E2) epoxide in vitro after heating

<table>
<thead>
<tr>
<th>Group</th>
<th>Nuclear RNA synthesis (pmol [32P]-GMP incorporated/mg DNA)</th>
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<tbody>
<tr>
<td></td>
<td>Before heating</td>
</tr>
<tr>
<td>Control</td>
<td>768 ± 9</td>
</tr>
<tr>
<td>Olive oil</td>
<td>768 ± 10</td>
</tr>
<tr>
<td>Sunflower oil</td>
<td>750 ± 16</td>
</tr>
<tr>
<td>Corn oil</td>
<td>791 ± 32</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>742 ± 18</td>
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<tr>
<td>Grapeseed oil</td>
<td>745 ± 43</td>
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</tbody>
</table>

Values given are the mean of 2–3 independent experiments. GMP, guanosine 5-monophosphate.
TAM in breast cancer therapy. However, this single mode of action of TAM is inadequate in explaining the fact that TAM is also known to induce endometrial39–42 and possibly other43–45 cancers. Recent studies indicate that after metabolic activation, TAM is able to bind to DNA, forming DNA adducts.45 These results strongly suggest that TAM is not only a carcinogen but, more specifically, an initiating carcinogen. Based on these facts, it is believed that TAM has at least two opposing mechanisms of action: (i) competing with E2 at the receptor level and blocking the promotional role of E2 in breast cancer; and (ii) binding to DNA after metabolic activation and initiating carcinogenesis. However, this dual mechanism of TAM action is still not able to explain how it is able to prevent breast cancer.39

17β-Estradiol requires activation by epoxidation to bind to DNA and form DNA adducts,24–26,28–30 as does TAM13 (Tables 2,3). This raises the possibility that TAM, as an effective competitor for epoxidation, may act indirectly by preventing the formation of E2 epoxide and, consequently, breast cancer. Our recent studies (Figs 8,9) have indeed shown that when incubated together with E2 for epoxidation, TAM is able to dramatically reduce the formation of E2 epoxide, as measured by both the loss of the ability of E2 to inhibit nuclear RNA synthesis and the reduced binding of [3H]-labelled E2 to nuclear DNA. Identical results were obtained when TAM and E1 were used. These results strongly suggest that the prevention of E2 epoxide formation through competitive epoxidation is the underlying mechanism used by TAM for its preventive effect against breast cancer.

Conclusions
Evidence has been presented to show that after activation by epoxidation, E2 is able to inhibit DNA-dependent RNA synthesis and bind to DNA, forming DNA adducts, both in vitro and in vivo. These experimental results not only lend strong support to our hypothesis regarding E2 epoxidation and the initiation of breast cancer, but also provide a molecular basis to screen potential chemopreventive agents against breast cancer. As shown in screening for the preventive potentials of vegetable oils and in deciphering the underlying mechanism of TAM for its preventive effect against breast cancer, the basic screening test determines whether a chemical agent is able to prevent the formation of E2 epoxide (i.e., prevention at the initiation step), as measured by both the loss of the ability of E2 to inhibit nuclear RNA synthesis and the reduced binding of [3H]-labelled E2 to nuclear DNA.

Table 2. Inhibition of 17β-estradiol (E2) epoxide on nuclear RNA synthesis in vitro

<table>
<thead>
<tr>
<th>Group</th>
<th>Nuclear RNA synthesis (pmol [32P]GMP incorporated/mg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>768 ± 9</td>
</tr>
<tr>
<td>E2</td>
<td>829 ± 15</td>
</tr>
<tr>
<td>E2 epoxide</td>
<td>200 ± 8</td>
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</tbody>
</table>

Values given are the mean of 3–4 independent experiments. GMP, guanosine 5-monophosphate.

Table 3. Effect of tamoxifen (TAM) and TAM epoxide on nuclear RNA synthesis in vitro

<table>
<thead>
<tr>
<th>Group</th>
<th>Nuclear RNA synthesis (pmol [32P]GMP incorporated/mg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>610 ± 16</td>
</tr>
<tr>
<td>TAM</td>
<td>491 ± 18</td>
</tr>
<tr>
<td>TAM epoxide</td>
<td>427 ± 18</td>
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</table>

Values given are the mean of 3–4 independent experiments. GMP, guanosine 5-monophosphate.
DNA-dependent RNA synthesis and the ability of [3H]-labelled E2 to bind to nuclear DNA. We believe that this screening technique will provide a fast and economical way to identify a wide variety of potential chemopreventive agents for further in vivo animal testing.

References


