Activation of 17β-estradiol and estrone by dimethyldioxirane and inhibition of rat liver nuclear and nucleolar RNA synthesis in vitro

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17β-estradiol (E₂), estrone and diethylstilbestrol (DES) had no effect on nuclear and nucleolar RNA synthesis in vitro. However, after reacting with dimethyldioxirane (DMDO), a versatile epoxide-forming oxidant, these estrogens were able to inhibit and in a dose-dependent manner nuclear and nucleolar RNA synthesis in vitro. It was also found that the time required for the maximal activation of these chemicals by DMDO varied: estrone, 10 min; E₂, 30 min; DES, 60 min. Tamoxifen (TAM) was also able to inhibit nuclear and nucleolar RNA synthesis in a dose-dependent manner, but the mechanism of this inhibition was more complex. Control experiments clearly indicated, unlike E₂, estrone and DES, TAM per se was able to directly inhibit RNA synthesis in vitro. TAM after activation by DMDO was able to further inhibit RNA synthesis contributing part of the total observed inhibition. These data show for the first time that E₂, estrone, DES and TAM can be activated by DMDO and possibly to epoxides. We propose that epoxidation of E₂ and estrone may be the underlying mechanism of carcinogenesis for these estrogens in vivo.

Introduction

Breast cancer leads all cancer incidence among American women, accounting for 32% of the 1995 estimated new cases in the United States. It is the second leading cause of cancer deaths, estimated at 46 000 per year (1). Estrogens, natural or synthetic, and the anti-estrogen tamoxifen (TAM*), used widely in a variety of clinical conditions, from estrogen replacement therapy to cancer treatment, are themselves carcinogenic, causing uterine (2,3), liver (4-7) and breast cancers (8-10). However, the mechanism of their carcinogenic action is still not well understood. Because estrogens are required for the growth and development of target cells, it has long been believed that estrogens are promoters for carcinogenesis (4,11,12). Recent studies have demonstrated that diethylstilbestrol (DES) (13,14) and TAM (15-22) are activated by the microsome P450 enzymes to epoxides that are able to bind DNA, forming DNA adducts. These findings suggest that DES and TAM, like other well established DNA-adduct forming chemical carcinogens (23-29), may be initiators for carcinogenesis. No information is presently available as to whether 17β-estradiol (E₂) and estrone can be activated to epoxides. Since these endogenous estrogens, like DES and TAM, are known to be carcinogenic (4,10-12), a hypothesis is therefore formulated and presented in Figure 1. Pathway A is the well established classical pathway depicting estrogens after binding to the receptors exert their effects in the stimulation of the growth and differentiation of cells in the target sites (30-36). For this reason, pathway A can be considered the normal physiological pathway. Pathway B, on the other hand, represents an alternative outcome for the estrogens in the dynamic, competitive, cellular environment. The hypothesis suggests that when the estrogens are not bound to the receptors, presumably due to either an excessive level of hormones or a lack of sufficient receptors present, these free estrogens are metabolized by the microsomal P450 enzymes to epoxides that, in turn, find their way to interact with DNA in the nucleus forming estrogen-DNA adducts to initiate carcinogenesis. This pathway is postulated to be the pathological pathway.

Using the versatile epoxide-forming oxidant, dimethyldioxirane (DMDO) (37), this paper reports the evidence that E₂ and estrone, like DES and TAM, can be activated and possibly to epoxides, and that these activated estrogens are strong inhibitors of nuclear and nucleolar RNA synthesis in vitro. In contrast, both E₂ and estrone per se have no effect on RNA synthesis. These studies therefore lend support to the proposed hypothesis that epoxidation of E₂ and estrone is a potential mechanism of carcinogenesis for these endogenous estrogens.

Materials and methods

Materials

17β-estradiol (E₂), estrone, diethylstilbestrol (DES), and tamoxifen (TAM), and aflatoxin B₁ (AFB₁) were all purchased from Sigma Chemical Company (St Louis, MO).

Activation of E₂, estrone, TAM and AFB₁ epoxides by dimethyldioxirane

The procedure using the specific epoxide-forming oxidant DMDO for the activation of E₂, estrone, DES and TAM was essentially the same as previously described by Yu et al (38). DMDO was prepared according to Adam et al (37). Briefly, 1.5 equivalent (2-3 ml) of the freshly prepared DMDO was mixed individually with 2 mg each of the chemicals and reacted at room temperature for 60 min with gentle shaking for the maximal activation, except in Figure 2 where the incubation time varied as indicated. After incubation, the reaction mixture was vacuumed to dryness and was redissolved in dimethylsulfoxide (DMSO) before use.

Rat liver nuclei and nucleoli isolations

Rat liver nuclei were isolated by the hypertonic sucrose method as described previously (39). Nucleoli were obtained by sonication of the isolated nuclei followed by discontinuous sucrose gradient centrifugation (40).

Incubation of the activated estrogens with the isolated nuclei and nucleoli and assay of nuclear and nucleolar RNA synthesis in vitro

Various amounts of each of the activated estrogens in DMSO were mixed with either 0.1 ml nuclei (150 μg DNA) or 0.1 ml nucleoli (15 μg DNA) in

Fig. 1. Epoxidation of estrogen as the basis for carcinogenesis.

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of these chemicals varied. For estrone, it required only 10 min for the maximal activation. This was indicated by the fact that its ability to inhibit nuclear RNA synthesis reached maximal 10 min after reacting with DMDO. Under identical conditions, E2 required 30 min for the maximal activation. DES and TAM needed 1 h to reach the plateau (2 h data are not shown). Aflatoxin B1 (AFB1) was used as a control. As shown also in Figure 2, it required only 10 min, as estrone, for the maximal activation.

The results in Figure 2 also suggest that there are differences in the ability of these activated estrogens to inhibit rat liver nuclear RNA synthesis. To confirm this observation, a dose-response inhibition experiment was carried out. For this experiment, E2, estrone, DES and TAM were all pre-treated for 1 h with 1.5 equivalent of DMDO to ensure the maximal activation of these chemicals. Then, different amounts of these activated estrogens were used for the testing of inhibition of nuclear RNA synthesis. As shown in Figure 3, when 50, 100, 150 and 200 µg of the activated E2 and estrone were used to react with rat liver nuclei (150 µg DNA) at room temperature for 10 min and assayed for RNA synthesis, the inhibition was similar for both activated E2 and estrone ranging from 20–25% inhibition at 50 µg to 70–80% inhibition at 200 µg dose levels. In contrast, the activated TAM and DES were able to inhibit only 37 and 28%, respectively, of the nuclear RNA synthesis even at 200 µg dose level.

The inhibition by these activated estrogens on rat liver nuclear (ribosomal) RNA synthesis was investigated. Figure 4 shows that the activated E2, estrone and DES were all able to strongly and in a dose-dependent manner inhibit the isolated rat liver nuclear RNA synthesis in vitro. The inhibition was similar for all three activated estrogens showing 35, 50 and 70% inhibition at 25, 50 and 100 µg dose levels, respectively.

Throughout these investigations, the inhibitory effect of these activated chemicals on nuclear and nucleolar RNA synthesis was always based on and compared with the corresponding individual untreated chemical controls. We noticed that unlike E2, estrone and DES, TAM per se was able to...
Estrogen activation and inhibition of RNA synthesis

**Fig. 4.** Dose-response inhibition curves of the DMDO activated 17β-estradiol, estrone and diethylstilbestrol on rat liver nucleolar RNA synthesis. The preparations of the various DMDO activated estrogens and the assay for RNA synthesis were the same as described in Figure 2, except all of the chemicals were pre-treated for 1 h with DMDO, and different doses of the activated chemicals as indicated were used to react with 0.1 ml nucleoli (15 μg DNA) for the assay of inhibition of nucleolar RNA synthesis, 17β-estradiol, O-O; estrone, x-x; diethylstilbestrol, □-□. Values given are means of two separate experiments.

**Fig. 5.** Dose-response inhibition curves of tamoxifen and DMDO activated tamoxifen on rat liver nucleolar RNA synthesis. The preparation of the DMDO activated tamoxifen and the assay for RNA synthesis were the same as described in Figure 2, except tamoxifen was pre-treated for 1 h with DMDO, and different doses of the activated tamoxifen were used to react with 0.1 ml nucleoli (15 μg DNA) for the assay of inhibition of nucleolar RNA synthesis. In a parallel study, untreated tamoxifen at different doses was used directly for the inhibition of nucleolar RNA synthesis. Tamoxifen, O-O; DMDO activated tamoxifen, ●-●. Values given are means of two to three separate experiments.

**Table I.** Direct inhibition of tamoxifen on rat liver nucleolar RNA synthesis in vitro

<table>
<thead>
<tr>
<th>Group</th>
<th>Nucleolar RNA synthesis (pmol [32P] GMP incorporated/mg DNA)</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8576±140</td>
<td>100</td>
</tr>
<tr>
<td>17β-estradiol</td>
<td>8605±424</td>
<td>100</td>
</tr>
<tr>
<td>Estrone</td>
<td>7712±351</td>
<td>90</td>
</tr>
<tr>
<td>Diethylstilbestrol</td>
<td>9272±615</td>
<td>108</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>4029±430</td>
<td>47</td>
</tr>
</tbody>
</table>

Various estrogens (100 μg) in 25 μl dimethylsulfoxide were incubated with rat liver nucleoli (15 μg DNA) in 100 μl 0.34 M sucrose at room temperature for 10 min. Control contained only 25 μl dimethylsulfoxide. At the end of the preincubation, RNA synthesis was assayed in 0.5 ml assay medium containing 1 μCi [α-32P] GTP at 37°C for 10 min. Values given are means ± SE of two to four experiments.

Discussion

17β-estradiol and estrone are endogenous female hormones and are also carcinogenic (4,10–12), causing increased risks of breast (9,10,45,46) and endometrial (47,48) cancers in women. At present, the mechanism(s) of their carcinogenic
action is not well understood. Since estrogens are required for growth and development in target cells, they have long been considered as promoters for carcinogenesis (4,11,12). Based on the recent findings showing that DES (13,14) and TAM (15–22) can be activated to epoxides and are able to bind DNA, we propose, as shown in Figure 1, that the natural estrogens, e.g. E₂ and estrone, under favorable conditions in vivo can also be activated to epoxides and to initiate carcinogenesis. To lend support to the proposed hypothesis, evidence is presented in this report showing for the first time that E₂ and estrone can be activated in vitro by the versatile epoxide-forming oxidant, DMDO. Although it is believed that the activated E₂ and estrone are most likely to be 17β-estradiol-1,2-epoxide, and estrone-1,2-epoxide, respectively, clearly, the final determination of these chemical structures has to be waited for in subsequent studies.

Another important feature of the proposed hypothesis is the belief that excessive level of free or unbound estrogens is a prerequisite for the epoxidation process to occur. In this regard, it is most interesting to find the recent reports showing that increased serum concentrations of endogenous estrogens are associated with an increase in breast cancer incidence (10,45), and that the exposure to greater than physiological estrogen concentrations due to ovarian stimulation correlates with the high risk of breast cancer (46).

Our data show that the kinetics for the maximal activation of E₂, estrone, DES and TAM varied (Figure 2). Estrone appeared to be the fastest, requiring only 10 min. DES and TAM took at least 1 h to reach the plateau under the used experimental conditions. We believe that this is related to the intrinsic chemical structures of these compounds. Thus, it is interesting to note that E₂ differs from estrone in having a hydroxyl group at C-17 instead of a ketone, the hydroxyl group apparently slowed down the activation process substantially. There are also differences in the ability of these activated chemicals to inhibit nuclear (Figure 3) and nucleolar (Figure 4) RNA synthesis. The data clearly indicate that the activated E₂ and estrone are much more effective in inhibiting nuclear RNA synthesis than DES and TAM. However, we found that the activated DES is equally effective in inhibiting nucleolar RNA synthesis as E₂ and estrone. The reason for this is not clear.

The mechanism of TAM inhibition of RNA synthesis is not a simple one. Clearly, TAM is by itself able to directly inhibit nucleolar RNA (Table I and Figure 5). This property is basically different from E₂, estrone and DES. These latter chemicals are entirely inactive in inhibiting RNA synthesis before activation by DMDO (Table I). The mechanism of this direct action of TAM may be due to either a direct interaction with DNA causing impairment of the DNA template function, or a specific inhibition of the RNA polymerase activity per se. Further experiments should clarify this uncertainty.

Also, it is interesting to note that AFB, a known potent carcinogen (49), was very easily activated by DMDO (Figure 2), and the activated AFB was very effective in the inhibition of nuclear and nucleolar RNA synthesis (Figure 6). When compared with E₂ and estrone, it is clear that the activated AFB is at least 200 to 1000 times more potent in inhibiting RNA synthesis either on a weight or molar basis (compare Figures 3 and 4 with Figure 6). If the ability of an activated chemical carcinogen to inhibit DNA-dependent RNA synthesis is a reflection of the potency of the carcinogen (50), E₂ and estrone, by comparison, are mild carcinogens. For clarification, it should be pointed out that previous studies have clearly shown that the product of AFB, activation by DMDO is AFB-8,9-epoxide (41), and that the DMDO activated AFB₁ is able to bind DNA and to inhibit DNA-dependent RNA synthesis (38).

Finally, it is important to mention that estrone-3,4-quinone has been proposed to be the tumor-initiating agent after estrone activation and that the free radicals generated by the metabolic redox cycling between quinone and hydroquinone forms of estrone are the basic mechanism for carcinogenesis (51–53). Also it should be mentioned that a recent report has concluded that in contrast to the mammary carcinogen, trans-3,4-dihydroxy-anti-1,2-epoxy-1,2,3,4-tetrahydrobenzo[c]phenanthrene, estrone-3-4-quinone was unable to produce tumors of the mammary gland, 44 weeks after it was injected under the nipples of a group of female rats (54).

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References
chromatography of a tamoxifen epoxide-deoxyguanylic acid adduct with a major DNA adduct formed in the livers of tamoxifen-treated rats. *Carcinogenesis*, 15, 793-795.


