Protective Effect of Dehydroepiandrosterone against Aflatoxin B₁- and 7,12-Dimethylbenz(a)anthracene-induced Cytotoxicity and Transformation in Cultured Cells¹

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SUMMARY

Dehydroepiandrosterone, an adrenal steroid that has been reported to be produced in subnormal amounts and found in lower plasma concentrations in women with benign and malignant breast tumors, protects cultured rat liver epithelial-like cells against aflatoxin B₁-induced cytotoxicity and protects hamster embryonic fibroblasts against aflatoxin B₁- and 7,12-dimethylbenz(a)anthracene-induced transformation. Related androgenic steroids show significantly less protective effect. Dehydroepiandrosterone also inhibits the rate of conversion of [³H]-7,12-dimethylbenz(a)anthracene to water-soluble metabolites by rat liver epithelial-like cells, suggesting that the steroid probably exerts its protective effect by inhibiting carcinogen activation.

INTRODUCTION

Several investigators have reported that women with breast cancer excrete significantly less of the androgen metabolites, androsterone and etiocholanolone, than do healthy control subjects (6, 7, 18). A prospective study on a large population of women ostensibly free of breast cancer has shown that the urinary excretion rates of androsterone and etiocholanolone are subnormal in individuals who subsequently develop the disease, with a linear increase in the risk of developing mammary cancer as the excretion rate of etiocholanolone fell from 1 mg/24 hr to 500 µg/24 hr (5).

Urinary androsterone and etiocholanolone are derived principally from 4 plasma precursors, dehydroepiandrosterone, dehydroepiandrosterone sulfate, androstenedione, and testosterone (21). It has been found that the urinary production rates of dehydroepiandrosterone and dehydroepiandrosterone sulfate were significantly lower in breast cancer patients than in control women, while the production rates of androstenedione and testosterone showed no significant difference (28). Similarly, premenopausal women with benign breast tumors, who are reported to have a greater probability of subsequently developing breast cancer (24), have been found to have significantly lower plasma concentrations of dehydroepiandrosterone sulfate than do matched controls (3).

A possibly similar hormone abnormality has been demonstrated in men with lung (29) and prostate cancer (23), who are reported to excrete significantly less androgrenone than matched controls, as well as in men and women with a variety of types of cancer (31), who have been found to have significantly lower plasma concentrations of dehydroepiandrosterone sulfate than control individuals.

Our results reported here demonstrate a protective effect of dehydroepiandrosterone against aflatoxin B₁-induced cytotoxicity and aflatoxin B₁- and DMBA²-induced transformation in cultured cells that is greater than that observed with related androgenic steroids.

MATERIALS AND METHODS

Aflatoxins B₁, B₂, G₁, and G₂ were obtained from Calbiochem, LaJolla, Calif., and DMBA was from Eastman Organic Chemicals, Rochester, N. Y. These compounds were prepared as stock solutions in DMSO (Fisher Scientific Co., Pittsburgh, Pa.) and were added to cultured cells to give a maximum DMSO concentration of 0.014%. Control cultures received DMSO without carcinogen.

Steroids. Androstenedione was obtained from Sigma Chemical Co., St. Louis, Mo., and etiocholanolone was from Research Steroids Laboratories, Inc., Penville, N. J., and from Steraloids, Inc., Pawling, N. Y. All other steroids were obtained from Steraloids, Inc. Steroids were prepared as stock solutions in ethanol and added to cell cultures to give a final ethanol concentration of either 0.07 or 0.17%. Control cultures received ethanol without steroid. Neither this concentration of ethanol nor of DMSO (above) had any apparent effect on the rate of acid-insoluble [³H]thymidine incorporation or cell growth.

Liver Epithelial-like Culture. A rat liver epithelial-like cell line (E-3) was established from an adult male Sprague-Dawley rat and propagated in monolayer culture in minimal essential medium (12) supplemented with 10% fetal calf serum, and buffered with either 10 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid- or 5 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid-5 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, adjusted to pH 7.6 (13). This line has undergone approxi-

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epithelial morphology. It is apparently similar to liver epithelial-like cultures that several other investigators have reported isolating, and that have retained few liver-specific functions (10, 33). However, unlike many established rodent fibroblast lines, it has retained its sensitivity to the cytotoxicity of the polycyclic hydrocarbons.

**[3H]Thymidine Incorporation.** E-3 cells (200,000 to 250,000) were inoculated into T-15 flasks and fed every 1 to 2 days. After 5 days of growth, cultures received a specific aflatoxin (B1, B2, G1, or G2) or, in the steroid experiments, either aflatoxin B1, a specific steroid, or the 2 in combination, and were incubated for approximately 24 hr at 37°. Following this, the medium was aspirated from the cells, and the cultures were washed 1 time with normal medium and reincubated for 24 hr in normal medium. Duplicate or triplicate cultures then received [3H]thymidine (New England Nuclear, Boston, Mass.; specific activity, 20 Ci/mmmole) at a final concentration of 0.33 μCi/ml for 90 min, and the acid-insoluble radioactivity per 100 μg of protein was determined (9). Incorporation of acid-insoluble radioactivity was linear for at least 3 hr under these conditions.

**Effect of Aflatoxins and Steroids on Cell Growth.** E-3 cells (150,000 to 200,000) were inoculated into T-15 flasks. After 3 days of growth, cultures received either medium containing a specific aflatoxin or, in the steroid experiments, aflatoxin B1, a specific steroid, or the combination of steroid and aflatoxin B1. After approximately 24 hr of incubation at 37°, the medium was aspirated from the cells, and the cultures were washed 1 time in normal medium and incubated in normal medium, with refedding every 1 to 2 days. At various-day intervals, duplicate or triplicate cultures were collected and the protein content per flask was determined (27).

**Effect of Steroids on DMBA- and Aflatoxin-induced Transformation.** The transformation assay of Berwald and Sachs (2) and DiPaolo et al. (11) was used. Primary rat embryonic fibroblasts received 5000 R of X-irradiation and were plated at 60,000 cells in 60-mm culture dishes in 4 ml of Dulbecco’s modification of Eagle’s minimum essential medium with 10% fetal calf serum, and incubated at 37° in an atmosphere of 95% air and 5% CO2. The following day, a culture of primary Syrian hamster embryonic fibroblasts was trypsinized, and 800 cells (in 1 ml of medium) were added to culture dishes containing X-irradiated rat cells. Approximately 24 hr later, the medium was removed, and fresh medium containing either DMBA (7.5 × 10−3 μm), aflatoxin B1, (10−4 μm), a specific steroid (10−4 μm), or the combination of carcinogen and steroid was added for 24 hr. The media were then removed and fresh medium without carcinogen or steroid was added, with changes every 2 to 3 days. Eight days after the hamster cells were plated, the cultures were fixed with methanol, stained with Giemsa, and the number of transformed clones (exhibiting a random, criss-cross orientation of cells) and normal clones was determined.

**Metabolism of [3H]DMBA.** The procedure of Kouri et al. (17) was used. From 100,000 to 200,000 E-3 cells were inoculated into T-15 flasks and fed on alternate days. Five days after inoculation, cultures received medium containing a specific steroid (10−4 μm) or control medium with ethanol (0.07%). Following a 30-min incubation at 37°, cultures received [3H]DMBA (Amersham/Searle, Arlington Heights, Ill.; 13 Ci/mmmole; approximately 96% pure) at a final concentration of 10−8 m. The [3H]DMBA was dissolved in DMSO and added to the cells to give a final DMSO concentration of 0.01%. The concentration of [3H]DMBA used (10−8 μm) had no apparent cytotoxic effect. At various intervals, the medium was collected, the cultures were washed with 2 ml of cold Earle’s balanced salt solution (without phenol red), and the washes were pooled with the medium. Five ml of acetone and 5 ml of hexane were added to the pooled medium-wash mixture, which was shaken and centrifuged at 900 × g for 5 min. The aqueous phase was reextracted with 5 ml of hexane and centrifuged. Aliquots of the aqueous phase (0.2 ml) and of the combined organic phases (0.5 ml) were each dissolved in 20 ml of toluene-Triton X solution and counted in an Intertechnique scintillation counter. The radioactivity in the aqueous phase following incubation of medium in T-15 flasks without cells was subtracted as background for each of the aqueous points. The background radioactivity was approximately 10% of the combined radioactivity recovered in the aqueous and organic phases, and it did not increase with time of incubation over a 16-hr period. In the presence of E-3 cells, there was a progressive increase in the amount of radioactivity recovered in the aqueous phase with incubation time, with approximately 80% of the radioactivity appearing in the aqueous phase after 16 hr of incubation in the nonsteroid cultures.

**RESULTS**

**Cytotoxicity of Various Aflatoxins.** We have shown previously that, within a series of methyl- and ethyl-substituted benz(a)anthracenes of varying carcinogenicities, there was a good correlation between the reported carcinogenicity of a derivative and its capacity to inhibit [3H]thymidine incorporation in the E-3 cell line (30). A similar relationship is shown here for a series of aflatoxins. Aflatoxin B1 is the most toxic and carcinogenic agent in the rat, followed in activity by the G1 derivative. Aflatoxins B2 and G2, which lack the vinyl ether double bond in the terminal furan ring, show little toxic and carcinogenic activity (8, 34). Table 1 shows that in the E-3 cell line the B1 derivative is the most effective inhibitor of [3H]thymidine incorporation, followed by the G1 derivative. Aflatoxins B2 and G2 have little, if any, inhibitory effect. Similar results are obtained when cytotoxicity is measured by overall growth inhibition following 24 hr of exposure to a specific aflatoxin (Chart 1).

**Effect of Steroids on Aflatoxin-induced Cytotoxicity.** Chart 2 shows the protective effect of various androgenic steroids against the aflatoxin B1-induced depression in growth of E-3 cells. Cultures treated with aflatoxin B1 and dehydroepiandrosterone had mean protein contents that were significantly greater than the mean protein contents of cultures treated with aflatoxin B1 alone (p < 0.001 at Days 7, 8, 9, and 10), as well as significantly greater than the values for cultures treated with aflatoxin B1 and etiocholanolone (p < 0.005; Day 7; p < 0.01; Day 8; p < 0.05, Day 9; p < 0.025).
Table 1
Effect of various aflatoxins on [\(^{3}H\)]thymidine incorporation in E-3 cells

E-3 cells were inoculated into T-15 flasks, and after 5 days of growth were treated with the various aflatoxins for 24 hr, washed, and incubated in normal medium for an additional 24 hr. The cultures were then pulsed with [\(^{3}H\)]thymidine for 90 min, and the acid-insoluble radioactivity per 100 μg of cell protein was determined as described in "Materials and Methods."

<table>
<thead>
<tr>
<th>Aflatoxin Concentration of</th>
<th>0</th>
<th>(10^{-4}) M</th>
<th>(5 \times 10^{-4}) M</th>
<th>(10^{-3}) M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aflatoxin B&lt;sub&gt;1&lt;/sub&gt;</td>
<td>3800 ± 180&lt;sup&gt;a&lt;/sup&gt;</td>
<td>135 ± 25</td>
<td>205 ± 6</td>
<td>632 ± 4</td>
</tr>
<tr>
<td>Aflatoxin B&lt;sub&gt;2&lt;/sub&gt;</td>
<td>3800 ± 180</td>
<td>2900 ± 165</td>
<td>3200 ± 105</td>
<td>3460 ± 375</td>
</tr>
<tr>
<td>Aflatoxin G&lt;sub&gt;1&lt;/sub&gt;</td>
<td>3800 ± 180</td>
<td>475 ± 8</td>
<td>753 ± 60</td>
<td>2025 ± 135</td>
</tr>
<tr>
<td>Aflatoxin G&lt;sub&gt;2&lt;/sub&gt;</td>
<td>3800 ± 180</td>
<td>3490 ± 210</td>
<td>3015 ± 20</td>
<td>3460 ± 470</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mean ± S.E.

Chart 1. Effect of aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, and G<sub>2</sub> on growth of E-3 cells. Three days after inoculation, cultured cells received medium containing a specific aflatoxin (O) or control medium (•) for 24 hr. Cells were then washed 1 time in normal medium and reincubated in normal medium with refueling every 2 to 3 days. Points are cell protein in duplicate (mean values) T-15 cultures. Aflatoxin concentrations (\(10^{-4}, 5 \times 10^{-4}, 10^{-3}\)) are molar.

Chart 2. Protective effect of dehydroepiandrosterone against aflatoxin B<sub>1</sub>-induced depression in growth of E-3 cells. Three days after inoculation, cultures received various test media for 24 hr. Cells were then washed 1 time in normal medium and reincubated in normal medium with refueling every 1 to 2 days. Points are cell protein of triplicate (mean values) T-15 cultures. The steroid concentration was \(10^{-3}\) M, and the aflatoxin B<sub>1</sub> concentration was \(4 \times 10^{-4}\) M.

Chart 3. Effect of dehydroepiandrosterone on the rate of metabolism of [\(^{3}H\)]DMBA to water-soluble products by E-3 cells. E-3 cells (130,000) were inoculated into T-15 flasks and fed on alternate days. On Day 5, cultures received medium containing either dehydroepiandrosterone or etiocholanolone (\(10^{-3}\) M) or ethanol (0.07%). Following 30-min of incubation at 37°, each culture received 0.4 M Ci of [\(^{3}H\)]DMBA (\(10^{-4}\) M), and at 3-, 6-, and 16-hr intervals after [\(^{3}H\)]DMBA addition, the medium from each culture was collected, and the amount of [\(^{3}H\)]DMBA converted to water-soluble metabolites was determined as described in "Materials and Methods." Each point is the mean value (± S.E.) of the amount of [\(^{3}H\)]DMBA converted to water-soluble metabolites for 4 separately treated cultures.

Day 9: \(p < 0.025\), Day 10) and aflatoxin B<sub>1</sub> and testosterone \((p < 0.01, \text{Day 7}; p < 0.001, \text{Day 8}; p < 0.005, \text{Day 9}; p < 0.001, \text{Day 10})\). Table 2 shows the protective effect of various androgenic steroids against the aflatoxin B<sub>1</sub>-induced depression in acid-insoluble [\(^{3}H\)]thymidine incorporation in E-3 cells. Here, also, dehydroepiandrosterone shows significantly greater protective activity than do the related androgenic steroids.
Protective Effect of Dehydroepiandrosterone

Table 2
Protective effect of androgenic steroids on aflatoxin-induced depression in [³H]thymidine incorporation in E-3 cells

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Total no. of clones</th>
<th>Cloning efficiency (%)</th>
<th>Transformed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Androstenedione</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>776</td>
<td>3.9</td>
<td>35</td>
</tr>
<tr>
<td>Dehydroepiandrosterone</td>
<td>1126</td>
<td>3.8</td>
<td>25</td>
</tr>
<tr>
<td>Testosterone</td>
<td>980</td>
<td>3.0</td>
<td>42</td>
</tr>
<tr>
<td>Etocholanolone</td>
<td>1289</td>
<td>4.3</td>
<td>40</td>
</tr>
<tr>
<td>Aflatoxin</td>
<td>876</td>
<td>1.9</td>
<td>237</td>
</tr>
<tr>
<td>Aflatoxin-dehydroepiandrosine</td>
<td>1060</td>
<td>2.8</td>
<td>103</td>
</tr>
<tr>
<td>Aflatoxin-testosterone</td>
<td>804</td>
<td>2.3</td>
<td>235</td>
</tr>
<tr>
<td>Aflatoxin-etiocholanolone</td>
<td>950</td>
<td>2.1</td>
<td>159</td>
</tr>
<tr>
<td>Dehydroepiandrosterone</td>
<td>1080</td>
<td>2.1</td>
<td>159</td>
</tr>
</tbody>
</table>

Table 3
Effect of androgenic steroids on aflatoxin B₁-induced transformation

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Total no. of clones</th>
<th>Cloning efficiency (%)</th>
<th>Transformed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aflatoxin-dehydroepiandrosine</td>
<td>1060</td>
<td>2.8</td>
<td>103</td>
</tr>
<tr>
<td>Aflatoxin-testosterone</td>
<td>804</td>
<td>2.3</td>
<td>235</td>
</tr>
<tr>
<td>Aflatoxin-etiocholanolone</td>
<td>950</td>
<td>2.1</td>
<td>159</td>
</tr>
</tbody>
</table>

Table 4
Effect of androgenic steroids on DMBA-induced transformation

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Total no. of clones</th>
<th>Cloning efficiency (%)</th>
<th>Transformed</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>419</td>
<td>2.6</td>
<td>7</td>
</tr>
<tr>
<td>Dehydroepiandrosterone</td>
<td>374</td>
<td>2.3</td>
<td>7</td>
</tr>
<tr>
<td>Testosterone</td>
<td>478</td>
<td>3.0</td>
<td>7</td>
</tr>
<tr>
<td>DMBA</td>
<td>403</td>
<td>1.7</td>
<td>70</td>
</tr>
<tr>
<td>DMBA-dehydroepiandrosterone</td>
<td>438</td>
<td>2.1</td>
<td>38</td>
</tr>
<tr>
<td>DMBA-testosterone</td>
<td>329</td>
<td>1.5</td>
<td>46</td>
</tr>
</tbody>
</table>

Effect of Steroids on Aflatoxin- and DMBA-induced Transformation. As shown in Table 3, dehydroepiandrosterone protects against aflatoxin B₁-induced transformation in cultured embryonic hamster fibroblasts. Some protection is also seen with etiocholanolone, while testosterone has no apparent activity. A similar protective effect of dehydroepiandrosterone against DMBA-induced transformation is shown in Table 4.

Metabolism of [³H]DMBA to Water-soluble Products. Treatment of E-3 cells with dehydroepiandrosterone produces a very significant reduction in the rate of conversion of [³H]DMBA to water-soluble metabolites (Chart 3). The amount of [³H]DMBA (mean value for 4 cultures) metabolized to water-soluble products was significantly less in the dehydroepiandrosterone cultures versus the nonsteroid cultures (p < 0.001, 3 hr; p < 0.001, 6 hr; p < 0.005, 16 hr) as well as in the dehydroepiandrosterone cultures versus the etiocholanolone cultures (p < 0.005, 3 hr; p < 0.001, 6 hr; p < 0.005, 16 hr).

Epiandrosterone, an analog of dehydroepiandrosterone that lacks the Δ₆,₈ double bond, was found to be a still more effective inhibitor of metabolism of [³H]DMBA to water-soluble products (Chart 4; epiandrosterone versus dehydroepiandrosterone cultures, p < 0.01, 3 hr; p < 0.001, 6 hr; p < 0.001, 16 hr).

DISCUSSION

Considerable evidence indicates that both DMBA (16) and aflatoxin B₁ (15, 32) must undergo metabolic activation by an NADPH-requiring mixed-function oxizide to their cytotoxic and carcinogenic metabolites. The inhibition by dehydroepiandrosterone of the rate of metabolism of [³H]DMBA to water-soluble products suggests that the steroid may exert its protective effect by interfering with carcinogen activation.
It appears well established that dehydroepiandrosterone is a potent noncompetitive inhibitor of mammalian glucose 6-phosphate dehydrogenase (19, 20, 22, 26). Oertel and Benes (25) tested a large series of steroids for their capacity to inhibit this enzyme and found that dehydroepiandrosterone (K₁, 0.79 × 10⁻⁸ M) and epiandrosterone (K₁, 0.59 × 10⁻⁸ M) were the most effective inhibitors. Inhibition of glucose 6-phosphate dehydrogenase would reduce the activity of the hexose monophosphate shunt, which is the primary source of extra-mitochondrial NADPH production (14). Since NADPH is a necessary cofactor for the metabolic activation of DMBA and aflatoxin B₁, this enzyme may exert its protective effect by reducing the level of this cofactor. The observed greater capacity of epiandrosterone, which is also a more effective inhibitor of glucose 6-phosphate dehydrogenase, to inhibit metabolism of [³H]DMBA to water-soluble products supports this hypothesis. However, further work is needed to establish this hypothesis.

Subnormal urinary production rates and plasma concentrations of dehydroepiandrosterone appear to be the one steroid abnormality demonstrated in women with benign (3) and malignant (4, 28) breast tumors, and a similar abnormality may exist in men and women with several other types of cancer (31). It is possible that these clinical observations may be related to the protective effect of the steroid reported here.

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