Modulation of Hormonal Induction of Tyrosine Aminotransferase and Glucocorticoid Receptors by Aflatoxin B₁ and Sterigmatocystin in Reuber Hepatoma Cells

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ABSTRACT

Employing Reuber rat hepatoma cells, H4-II-E, the effects of aflatoxin B₁ (AFB₁) and sterigmatocystin (STC), which exhibit a similar cytotoxicity but a marked difference in hepatocarcinogenicity, on the hormonal induction of tyrosine aminotransferase (TAT), on glucocorticoid receptors, and on their nuclear acceptor sites were investigated. AFB₁ strongly inhibited hydrocortisone-inducible TAT activity. The IC₅₀ value was 0.2 µg/ml. AFB₁ also showed weak inhibitory effects on insulin- and dibutylryl cyclic AMP-inducible TAT activities. In contrast, the IC₅₀ of STC on hydrocortisone-inducible TAT activity was 3.5 µg/ml, about 10 times higher than that of AFB₁. Dibutylryl cyclic AMP- and insulin-inductions were not depressed by STC.

AFB₁, inhibited the formation of cytosolic glucocorticoid receptor-hormone complexes (GRCs) but STC did not. Moreover, AFB₁, activated in vitro by the microsomal cytochrome P-450 system, interfered more markedly in the formation of cytosolic GRCs than STC did. Sucrose density gradient analysis of GRCs and Scatchard analysis revealed that AFB₁ and STC mainly impaired glucocorticoid receptors and GRC-acceptor sites, respectively. The present data suggest a marked difference between AFB₁ and STC with regard to the inhibition of hormonal induction of liver specific enzymes.

INTRODUCTION

It has been recognized that AFB₁, produced by strains of Aspergillus flavus, exhibits hepatotoxic and hepatocarcinogenic activities (1). Its contamination in foodstuff and animal feed is of great concern as a health hazard. STC is also a dihydrobis-furanoid mycotoxin like AFB₁, and it possesses comparable cytotoxicity and mutagenicity to AFB₁ (2, 3), but its hepatocarcinogenicity is far less potent (4).

Biochemical alterations associated with AFB₁ toxicity in the liver and its metabolic pathway were studied extensively in several laboratories, as reviewed by Wogan et al. (1) and the authors (5, 6). During the past several years, we have extensively studied the metabolism of AFB₁, and related mycotoxins (7–10). The binding of AFB₁ to human DNA (11) and the activation of proto-oncogenes (12) were reported. Most recently, we have also demonstrated the over-expression of c-Ha-ras and c-myc oncogenes in AFB₁-induced hepatocellular carcinomas and in the established cell line, Kagura-1 (13, 14).

It is well established that gene expression and differentiation are regulated by many cellular factors and enzymes. Past reports on the mode of action of AFB₁ focused mainly on the interactions between this potent carcinogen and DNA (1, 8, 15). As for association with proteins, no detailed experiments were performed except the interactions with GR in nuclei (16), nuclear histone I (1) and RNA polymerase II (17).

With an aim to elucidate a possible interaction between this mycotoxin and cellular regulatory proteins, we investigated in detail the effects of AFB₁ on hormonal induction of TAT and GR. As is well known, glucocorticoids have been shown to play an important role in gene expression in the liver in vivo and cell lines of liver origin in vitro (18, 19).

With this in mind, we used Reuber rat hepatoma cells, H4-II-E, which possess many of the functional enzymes of mature liver cells, and compared the effects of AFB₁ with those of the weak hepatocarcinogen, STC, on TAT induction and modulation of the GR function.

MATERIALS AND METHODS

Cell Line, Media, Culture Conditions. The cells, H4-II-E, purchased from American Type Culture Collection (MD), were first grown to a monolayer in minimum essential medium (Nissui, Tokyo) supplemented with 5% fetal calf serum (GIBCO, OH) in a humidified atmosphere of 7% CO₂ and air. The following experiments were performed with the cells grown in the serum-free minimum essential medium for 16–18 h.

Enzyme Assay and Cellular Fractionation. After washing the cells with Ca²⁺- and Mg²⁺-free Dulbecco’s PBS(−), (Nissui, Tokyo) and then 0.125 M potassium phosphate buffer (pH 7.6), the cells were harvested by rubber policeman, and then homogenized in a Potter-Elvejhem type homogenizer. The lysates were assayed for TAT by the method of Granner and Tomkins (20). For the fractionation of cellular organella, the cells were washed with PBS(−) and then buffer AD, and the cellular lysates were centrifuged at 105,000 × g 1 h. The resulting supernatant was used as the cytosol. To obtain nuclei, the cells were washed with PBS(−) and buffer C. Five ml of the cellular lysates in buffer C were layered over 5 ml of 0.88 M sucrose-10 mM MgCl₂, then centrifuged at 1,200 × g 10 min, and the precipitates were suspended in buffer C, followed by centrifugation at 1,000 × g 10 min. Protein and DNA were estimated by the methods of Lowry et al. (21) and Burton (22) with BSA and calf thymus DNA as standards, respectively.

Cell-free Formation of GRCs and Their Transfer to Nuclei. Our methods for analysis of estrogen receptors (23) were adopted as follows. The cytosol was incubated with 75.5 mM [³H]TA in the presence and absence of AFB₁, or STC at 0°C for 2 h, and after an addition of 5% dextran-coated charcoal followed by centrifugation at 0°C for 1,200 × g × 15 min, the radioactivity in the supernatant was counted. For nonspecific binding, 100 times the amount of cold TA was added and the reaction was processed as above. For nuclear transfer, GRCs were activated by an incubation at 20°C for 30 min in buffer AD containing 0.15 M NaCl, and the resulting “activated GRCs” were diluted by the same volume of buffer AD and incubated with the nuclear fraction at 0°C for 1 h. The whole was mixed with 1 ml of buffer C, and centrifuged for 1,000 × g × 10 min. After washing with 1 ml of buffer C, the radioactivity in the precipitates was counted.

For the metabolic activation of the mycotoxins, the cells were washed with PBS(−) and homogenized in buffer AD, followed by centrifugation for 9,000 × g × 20 min. The resulting S-9 (375 µl) was mixed with 9 µl of AFB₁ (1 mg/ml) or 4.5 µl of STC (2 mg/ml) dissolved in DMSO...
and 15 µl of 75 mM NADPH. After an incubation at 20°C for 30 min and a subsequent centrifugation at 2°C for 105,000 x g x 1 h, the resulting cytosol was used for the assay of [3H]TA-GR complex formation.

Sucrose Density Gradient Analysis of GRCs. For sucrose density gradient centrifugation analysis of the cytosolic "nonactivated form," 5.0 µl of 10 mM antipain and 2.0 µl of 1 mM sodium molybdate were added to 200.0 µl of the cytosolic [3H]TA-GR (24), and 190.0 µl of the mixture was layered over 5 ml of 5-20% sucrose gradient solution in buffer AD containing 0.25 mM antipain and 10 mM sodium molybdate. After centrifugation for 40,000 rpm x 12.5 h, the distribution of radioactivity was determined.

For analysis of the "activated form" of GRC, 250 µl of the cytosolic [3H]TA-GRCs were mixed with 5.5 µl of 10 mM antipain and 20.6 µl of 1.65 M NaCl-buffer AD. After incubation at 20°C for 30 min, the aliquot (210 µl) was layered over 5 ml of 5-20% sucrose-0.15 M NaCl solution, followed by centrifugation for 40,000 rpm x 23 h. BSA (4.6 S) and human γ-globulin 7.0 S were used as marker proteins.

Kinetic Analysis. For the cytosolic GR, the cytosol was incubated at 0°C for 2 hr in the presence of various concentrations of [3H]TA in the range of 0.997-19.9 mm. After removal of free steroids by the dextran-charcoal method, the radioactivity in the cytosol was counted. The background level was subtracted by an addition of cold TA. For the nuclear acceptor sites, the "activated GRC" was mixed with various amounts of the nuclear fraction. After incubation at 0°C for 1 h, the reaction mixture was mixed with 1 ml of buffer C, and the whole was centrifuged for 1,000 x g x 10 min. After an additional centrifugation as above, the radioactivity in the precipitate was counted. The Kd values and numbers of binding sites were calculated by Scatchard plot analysis (25).

Chemicals and Enzymes. Bt2cAMP, insulin, and AFB1 were produced by Sigma (MS). [3H]TA (30 Ci/mmol) was produced by Amersham (IL). STC, the kind gift of Dr. Hamasaki (Tottori University), was purified before use. Antipain was a gift of Dr. Hazato (Tokyo Metropolitan Institute of Medical Sciences, Tokyo).

RESULTS

Basic Conditions for Hormonal Induction of TAT Activity. Before detailed analysis was made on the effects of mycotoxins, the induction of TAT activity in Reuber hepatoma cells, H4-II-E, was investigated with regard to the concentration of hormones and the time for induction. TAT activity increased sharply, paralleling the concentration of HC. The maximal activity, 4 times higher than in the control, was reached at 1.0 x 10^-7 M HC. TAT activity was also induced by insulin and Bt2cAMP. The time dependencies of TAT induction with 1.0 x 10^-7 M HC, 1.74 x 10^-7 M insulin, and 3.0 x 10^-3 M Bt2cAMP revealed that the maximal activity of TAT was observed 4 h after addition of insulin and Bt2cAMP, while the increase continued beyond 6 h after HC addition (data not shown).

From these results, the basic conditions for hormonal induction were fixed as follows: Incubation with 1.0 x 10^-7 M HC, 1.74 x 10^-7 M insulin, and 3.0 x 10^-3 M Bt2cAMP revealed that the maximal activity of TAT was observed 4 h after addition of insulin and Bt2cAMP, while the increase continued beyond 6 h after HC addition (data not shown).

Cytotoxicity and the Inhibition of TAT Induction. In order to determine the cytotoxicity of mycotoxins to H4-II-E cells, the cells were exposed to various concentrations of mycotoxins, and their survival rates were compared by trypan blue staining. Neither AFB1 nor STC exhibited any significant decrease in numbers of the survival cells at doses of 5-10 µg/ml when the cells were exposed for 6 h (Fig. 1).

Inhibitory effects of AFB1 and STC on the hormonal inductions of TAT activity are shown in Fig. 2. HC-induced TAT activity was markedly inhibited by AFB1, and the IC50 was estimated at 0.2 µg/ml. Insulin- and Bt2cAMP-dependent inductions were also dose-dependently inhibited by AFB1, and 20% and 40% inhibition were induced by 5 µg/ml of AFB1, respectively.

As for STC, HC-dependent induction of TAT activity was inhibited dose-dependently, and the IC50 was 3.5 µg/ml, about 10 times higher than that of AFB1. No significant depression of Bt2cAMP-dependent induction of TAT activity was observed. STC showed no inhibitory effects but stimulated rather slightly insulin inducement, as shown in Fig. 2.

Effects of the Mycotoxins on Formation of GRCs in a Cell-free System. To elucidate whether or not the mycotoxins possess a direct effect on the process of formation of GRCs, the cytosol of cells was incubated at 0°C with [3H]TA in the presence or absence of the mycotoxins. No decrease in the radioactivity of [3H]TA-bound GR was observed with up to 13 µg/ml of the mycotoxins added (data not shown). Moreover, neither the activation of cytosolic GRCs nor the transfer of "activated GRCs" to the nuclear acceptor sites was disturbed by up to 15 µg/ml of AFB1 and STC (data not shown).When AFB1 and STC were incubated with S-9 in the presence of NADPH prior to the complex formation process, the [3H]TA-

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Fig. 1. Cytotoxicities of AFB1 and STC on H4-II-E cells. The cells were incubated with various concentrations of AFB1 (C) or STC (B) or with DMSO as a control for 6 h. Viable cells were counted on a hemocytometer after staining with trypan-blue as described in "Materials and Methods." Each point was derived from experiments done in duplicate.

Fig. 2. Effects of AFB1, and STC on the hormonal induction of TAT in H4-II-E cells. The cells were incubated with 1.0 x 10^-7 M HC for 6 h (O), 3 x 10^-3 M Bt2cAMP for 4 h (△), 1.74 x 10^-7 M insulin for 4 h (□) and combined with DMSO or various concentrations of AFB1 (A) and STC (B). TAT activity was then determined. Each value was derived from experiments done in duplicate as described in "Materials and Methods." Percentage of hormone-inducible TAT activity was calculated as follows: (AFB1 or STC/hormone-treated/DMSO/hormone-treated) x 100.
Effects of aflatoxin B₁ and sterigmatocystin on GR

bound to GRs decreased to 78 and 89% of the control, respectively (Table 1).

Sucrose Density Gradient Analysis of GRCs. Using sucrose density gradient centrifugation analysis, the S values of nonactivated and activated forms of the cytosolic GRC were estimated to be 6.5 and 4.6, respectively (Fig. 3). AFB₁ induced no significant change in these S values, but the number of GRCs was reduced to about 65% of the control.

Further experiments with cycloheximide revealed that the AFB₁-induced reduction of cytosolic GRCs was not affected by 2.5 μg/ml of cycloheximide, which inhibited protein synthesis by more than 98% in the control (Fig. 4). As for the cells treated with 5 μg/ml of STC for 6 h prior to HC, significant changes were not observed in either the profiles of GRCs or in the transfer rate into the "active form" (Fig. 5).

Kinetic Analysis of GRCs. In order to elucidate the modulation of GRC by the mycotoxins, kinetic analysis on the cytosolic

Table 1  Effects of metabolically activated AFB₁ and STC on GRs

| S-9 fractions prepared from H4-II-E cells were incubated with 22.6 μg/ml of AFB₁ or STC in the presence of 3 mM NADPH. The reaction mixtures were centrifuged at 105,000 × g for 1 h, and the resulting supernatants were incubated with [3H]TA. [3H]TA-GR complexes were determined as described in "Materials and Methods." Each value was derived from experiments done in duplicate. |
|---|---|
| [3H]TA-receptor complexes (dpm) | % of control |
| 0°C, 30 min | 17,925 | 100 |
| 20°C, 30 min | 14,141 | 78 |
| + AFB₁ (22.6 μg/ml) | 10,953 | 62 |
| + STC (22.6 μg/ml) | 12,562 | 89 |

Fig. 3. Sucrose density gradient analysis of cytosolic [3H]TA-GR. The cytosols were prepared from the cells treated with (A) or without (B) 5 μg/ml of AFB₁ for 6 h. A, nonactivated; B, activated [3H]TA-GR.

Table 2  Kinetic analysis of cytosolic receptors and nuclear acceptor sites in cells treated with AFB₁ and STC

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Treatment</th>
<th>Kₛ (μM)</th>
<th>Sites (pmol/mg DNA)</th>
<th>Kₛ (μM)</th>
<th>Sites (pmol/mg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DMSO</td>
<td>3.1 × 10⁻⁶</td>
<td>0.55</td>
<td>1.4 × 10⁻¹⁰</td>
<td>0.85</td>
</tr>
<tr>
<td>2</td>
<td>AFB₁</td>
<td>4.7 × 10⁻⁶</td>
<td>0.18</td>
<td>1.0 × 10⁻¹⁰</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>STC</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>DMSO</td>
<td>2.0 × 10⁻⁶</td>
<td>ND</td>
<td>0.74 × 10⁻¹⁰</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>STC</td>
<td>2.2 × 10⁻⁶</td>
<td>0.61</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

*ND, not determined.
within nearly the same range of 0.85 pmol/mg DNA, while the $K_d$ values were about 30% lower than in the control.

In the cells treated with 5 µg/ml of STC for 6 h, the $K_d$ value of cytosolic GR fell within nearly the same range of $2.0 \times 10^{-9}$ M, and the number of GR sites increased to 122% of the control. While the nuclear acceptor sites and the $K_d$ values were about 33 and 45% lower than the controls, respectively.

**DISCUSSION**

It is well established that many fungi produce more than a hundred toxic metabolites (so-called mycotoxins) which are implicated in food-borne intoxications in humans and farm animals (5). Of the numerous mycotoxins, AFB$_1$ is extensively investigated, since this dihydro-bisfuranoid mycotoxin is the most potent carcinogen known thus far. It is closely associated with the development of human primary liver cancer, which is endemic in southern Asia and South Africa, where foods are heavily contaminated with AFB$_1$.

Current topics of cancer development are the covalent binding of AFB$_1$, with DNA after activation into AFB$_1$-8,9-oxide (1, 8, 15), and the subsequent expression of oncogenes in target organs (12-14). Since gene expression and cellular differentiation are regulated by several hormones and cellular factors, we attempted to demonstrate the possible alteration of the cellular regulatory system in Reuber hepatoma cells.

Our basic approach to the hormonal induction of liver-specific enzymes revealed that TAT activity was induced by various hormones such as HC, insulin, and Bt$_2$cAMP. The degree of their induced activities was closely related to the concentrations of hormones added and the exposure times (data not shown).

The concentrations of AFB$_1$ and STC were selected at 5–10 µg/ml, which gave rise to no significant changes of viability of Reuber hepatoma cells. Thus, there is no marked difference in the susceptibility to the bisfuranoïds, AFB$_1$, and STC (Fig. 1).

The data were presented clearly demonstrated that the inhibitory effect of AFB$_1$, on the HC-induced TAT activity, which accompanied the net synthesis of mRNA, was different from that of Bt$_2$cAMP-dependent induction of TAT, which was also known to accompany the synthesis of mRNA (26-28). Our preliminary experiments indicated that AFB$_1$ decreased the HC- and Bt$_2$cAMP-induced TAT mRNA levels in H4-II-E cells (data not shown). Therefore, it is conceivable that the effects of AFB$_1$ on the hormonal induction of enzymes was multiple, and that the inhibition observed was not simply limited to the activity of template DNA (1) and RNA polymerases (17).

As for STC, which possessed a similar cytotoxicity to AFB$_1$, a concentration 15 times higher than that of AFB$_1$ was required to inhibit the HC-dependent induction of TAT (Fig. 2). No significant effects were observed in the insulin- and Bt$_2$cAMP-inducible TAT activities (Fig. 2). Therefore, the mode of action of STC was presumed to be markedly different from that of AFB$_1$ in the hormonal induction of TAT activity in Reuber hepatoma cells.

Based on the observation that AFB$_1$ interfered with HC-dependent induction of TAT (Fig. 2), we supposed that there were very sensitive molecules for AFB$_1$ in the cascade of TAT induction by HC. The detailed mechanism of AFB$_1$-induced disturbance of HC-inducible TAT activity was then investigated.

In the cell-free system, GRC formation analysis proved no direct effect on the noncovalent binding of PHJTA to the cytosolic GR or their transfer to the nuclear acceptor sites.
hepatic cytochrome P-450 isoenzymes from PCB-treated rats and participation of cytochrome b, in the activation of aflatoxin B. Carcinogenesis (Lond.), 4:1071–1073, 1983.


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