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Inhibitory Effect of Sterigmatocystin and 5,6-Dimethoxysterigmatocystin on ATP Synthesis in Mitochondria

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The inhibitory effects of sterigmatocystin, *O*-methylsterigmatocystin, and 5,6-dimethoxysterigmatocystin on the ATP synthesis system in mitochondria were compared with that of aflatoxin B_1, which disturbs the respiratory chain in mitochondria. Sterigmatocystin and 5,6-dimethoxysterigmatocystin were found to uncouple the oxidative phosphorylation process without causing depression of state 3 respiration. *O*-Methylsterigmatocystin did not exhibit uncoupling activity at the limited concentrations tested (due to its low solubility in an aqueous system). These compounds, as well as aflatoxin B_1, elicited neither pseudo-energized nor energized swelling of mitochondria and did not inhibit Ca^{2+}-induced swelling of mitochondria.

Sterigmatocystin (Fig. 1), a xanthone derivative possessing a dihydrobisfuran ring, is chemically and metabolically related to aflatoxin B_1 and is toxic and carcinogenic, although it is less potent than aflatoxin B_1 (1, 5, 6, 8, 22). Aflatoxin B_1 interferes with ATP synthesis in mitochondria by interacting with the respiratory chain, which has been considered to be a part of a toxic consequence (3, 4). Secalonic acid D, a xanthone mycotoxin from *Aspergillus ochraceus* and *Penicillium oxalicum*, is an inhibitor to oxidative phosphorylation and to Ca^{2+}-induced swelling of mitochondria (10). Averufin and versicolorin A, which are biosynthetic precursors of sterigmatocystin and aflatoxin B_1 (1, 2, 8), have also been demonstrated to be extremely potent uncouplers of mitochondrial respiration (12, 13). Recently, 5,6-dimethoxysterigmatocystin has been isolated from *Aspergillus multicolor* (7) and has been found to be more toxic than sterigmatocystin (21). However, sterigmatocystin and 5,6-dimethoxysterigmatocystin have not been investigated for their effects on mitochondrial reactions. It is, therefore, of importance to examine the effects of these compounds on ATP synthesis in isolated mitochondria. To gain insight into the role of the hydroxyl group of the xanthone nucleus in the biological activities of these compounds, we examined the uncoupling effect of *O*-methylsterigmatocystin as well as that of sterigmatocystin.

MATERIALS AND METHODS

Reagents. Sterigmatocystin was isolated by Sekita et al. (18) from *Chaetomium thielavioideum*. 5,6-Dimethoxysterigmatocystin, originally isolated by Hamasaki et al. (7) from *A. multicolor*, was newly isolated by Maebayashi et al. from *Emericella foeniculicola*. *O*-Methylsterigmatocystin and aflatoxin B_1 were kindly donated by R. J. Cole, National Peanut Research Laboratory, U.S. Department of Agriculture, and by Y. Ueno, Tokyo University of Science, respectively. All compounds were dissolved in *N*,*N*-dimethylformamide. Bovine serum albumin was a product of Armour Pharmaceutical Co. (Kankakee, Ill.). ADP and Tris were purchased from Sigma Chemical Co. (St. Louis, Mo.). Other reagents were of the purest grade commercially available.

Procedures. Rat liver mitochondria were prepared basically by the procedure of Schneider (17), with a solution of 0.25 M sucrose. Mitochondrial respiration was measured by means of a Galvani-type oxygen electrode (Sensanics Japan Co., Tokyo). The reaction mixture was composed of 675 μmol of sucrose, 30 μmol of KCl, 15 μmol of $MgCl_2$, 15 μmol of phosphate, 60 μmol of Tris-chloride, 1.5 μmol of EDTA, and 3 mg of mitochondrial protein in a final volume of 3.0 ml (pH 7.4). The reaction was initiated by adding 15 μmol of succinate and was carried out at 24°C. Respiratory control (RC) index and P/O ratio were calculated from an oxygram by the method of Chance and Williams (2). Mitochondrial swelling was monitored by measuring the decrease of light absorption at 550 nm by the method of Tedeschi and Harris (19). The reaction medium contained 300 μmol of KCl, 40 μmol of Tris-chloride, and 0.9 mg of mitochondrial protein in a final volume of 2.0 ml (pH 7.4). In the control experiment, 20 μl of *N*,*N*-dimethylformamide was added instead of mycotoxin solution. Each reaction was performed in a glass cuvette which was placed in a Hitachi 320S recording spectrophotometer at 24°C. Mitochondrial protein was determined by the method of Lowry et al. (14) with bovine serum albumin as a standard protein.

RESULTS AND DISCUSSION

The oxygram of mitochondrial respiration oxidizing succinate is illustrated in Fig. 2. Freshly prepared mitochondria exhibited tightly coupled respiration, giving a high RC index and a high P/O ratio (Fig. 2, curve 1). The addition of sterigmatocystin to state 4 respiration (depressed respiration due to lack of ADP) caused marked decreases in RC index and P/O ratio (Fig. 2, curve 2), which indicates that sterigmatocystin exerted an uncoupling effect on oxidative phosphorylation in mitochondria. The rate of state 3 respiration (fully activated respiration by ADP) was not affected by this compound. Aflatoxin B_1 did not exhibit the uncoupling activity. State 4 respiration was not accelerated by aflatoxin B_1. State 3 respiration was depressed by the compound; only the RC index was diminished without an accompanying decrease in the P/O ratio (Fig. 2, curve 3).

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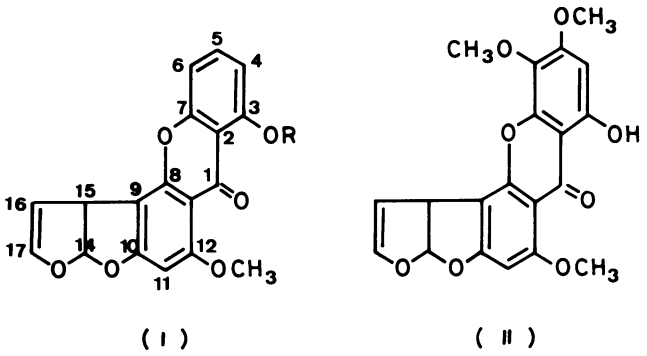

FIG. 1. Structures of sterigmatocystin, *O*-methylsterigmatocystin, and 5,6-dimethoxysterigmatocystin (II). (I) R = H, sterigmatocystin; R = CH_3, *O*-methylsterigmatocystin.

The uncoupling activities of sterigmatocystin, *O*-methylsterigmatocystin, and 5,6-dimethoxysterigmatocystin on mitochondrial respiration were compared (Fig. 3). Sterigmatocystin and 5,6-dimethoxysterigmatocystin decreased the RC index (Fig. 3, curves 3 and 2, respectively) without inhibiting the state 3 respiration (curves 5 and 6, respectively), indicating that both compounds are uncouplers. Sterigmatocystin was found to be apparently stronger than 5,6-dimethoxysterigmatocystin in uncoupling activity. *O*-Methylsterigmatocystin did not diminish the RC index at concentrations lower than 42 μM (Fig. 3, curve 1), indicating that this compound does not possess the uncoupling activity. Due to the low solubility of *O*-methylsterigmatocystin in an aqueous system, the experiment was not performed with *O*-methylsterigmatocystin at concentrations greater than 42 μM. Curve 4 of Fig. 3 shows the inhibitory effect of aflatoxin B_1 on state 3 respiration. Aflatoxin B_1 markedly depressed state 3 respiration by interaction with the respiratory chain

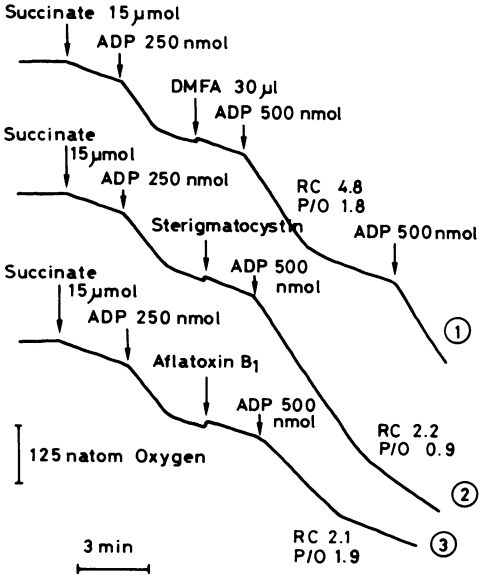

FIG. 2. Effects of sterigmatocystin and aflatoxin B_1 on mitochondrial respiration. Sterigmatocystin and aflatoxin B_1 were added at concentrations of 60 and 200 μM, respectively. Other detailed conditions and curves 1, 2, and 3 are described in the text. DMFA, *N*,*N*-dimethylformamide.

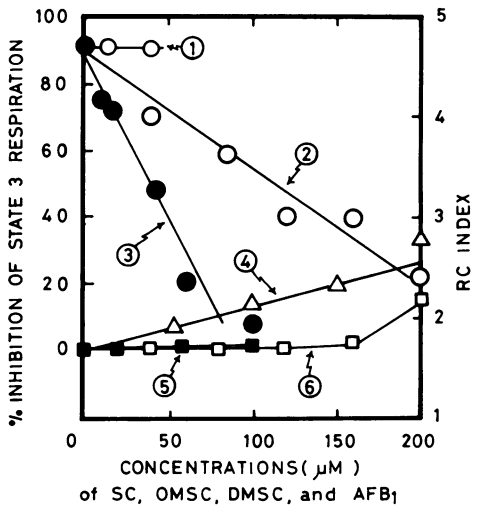

FIG. 3. Uncoupling effects of sterigmatocystin and 5,6-dimethoxysterigmatocystin. Reaction conditions are as described in the legend to Fig. 2. Curves 1, 2, and 3 show the effects of *O*-methylsterigmatocystin, 5,6-dimethoxysterigmatocystin, and sterigmatocystin on the RC index, respectively. Curves 4, 5, and 6 show the effects of aflatoxin B_1, sterigmatocystin, and 5,6-dimethoxysterigmatocystin on state 3 respiration, respectively.

(6, 7), which apparently indicates that the mode of action of sterigmatocystin is quite different from that of aflatoxin B_1.

These results indicate that sterigmatocystin and 5,6-dimethoxysterigmatocystin are uncouplers of oxidative phosphorylation and that the presence of the hydroxyl group on the xanthone nucleus is important for the uncoupling activity of either of these compounds. The molecular mechanism for their uncoupling activity may be identical with that of classic uncouplers such as 2,4-dinitrophenol and FCCP, which are proposed to uncouple mitochondrial respiration by acting as proton carriers across mitochondrial inner membranes (15, 16, 20). Sterigmatocystin was deprived of the proton conductivity by the *O*-methylation, by which the uncoupling activity was canceled, as observed for the uncoupling activities of xanthomegnin (9), emodin (11), skyrin (11), averufin (12),

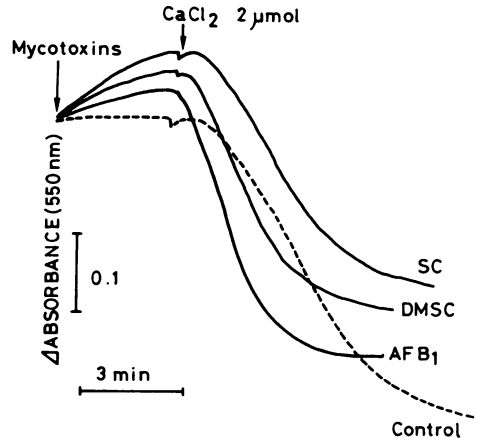

FIG. 4. Effects of sterigmatocystin and aflatoxin B_1 on Ca^{2+}-induced swelling of mitochondria in 0.15 M KCl solution. Detailed reaction conditions are described in the text. SC, Sterigmatocystin; DMSC, 5,6-dimethoxysterigmatocystin; and AFB_1, aflatoxin B_1.

and versicolorins A and B (13). From this viewpoint, it is reasonable to consider that aflatoxin B_1 does not possess an uncoupling activity due to its lack of a phenolic hydroxyl group. The uncoupling effects of sterigmatocystin and 5,6-dimethoxysterigmatocystin may cause a diminution in the rate of mitochondrial ATP synthesis which may interpret a part of the molecular mechanism for their cytotoxicities, though their potencies in the uncoupling effect are not strong enough for assessing in vivo toxicities. The reason is not clear why 5,6-dimethoxysterigmatocystin, which has a greater cytotoxic effect than sterigmatocystin (21), has a lower uncoupling activity. One would expect that the hydrophobicity of sterigmatocystin should be decreased by the methoxylation of the xanthone nucleus, by which the potency in the uncoupling activity might be decreased and, in reverse, the strength in the profound inhibitory effect on nucleus functions (22) was advanced.

Figure 4 shows the Ca^{2+}-induced swelling of mitochondria in 0.15 M KCl solution. Ca^{2+} is known to induce mitochondrial swelling by eliciting a rapid release of an endogenous uncoupling factor (23), causing a marked decrease in light absorption (19). Secalonic acid D, a xanthone mycotoxin from *A. ochraceus*, has been demonstrated to strongly impede the Ca^{2+}-induced swelling (9). As illustrated in Fig. 4, Ca^{2+} was able to induce mitochondrial swelling even in the presence of sterigmatocystin, 5,6-dimethoxysterigmatocystin, or aflatoxin B_1, indicating that these mycotoxins do not hinder the Ca^{2+}-induced swelling of mitochondria. The addition of these mycotoxins did not cause an abrupt decrease in absorbance but produced a small-scale absorbance increase in the absence of Ca^{2+}, indicating that these mycotoxins themselves do not induce mitochondrial swelling (pseudo-energized swelling) in isotonic KCl solution. They did not elicit mitochondrial swelling (energized swelling) in isotonic sucrose solution in the presence of succinate, a substrate for the respiratory chain (data not shown). The small-scale increase in absorbance produced by mycotoxins (Fig. 4) was also observed in the control experiment without mitochondria (data not shown), indicating that the absorbance increase is of an artifact probably due to the addition of highly hydrophobic compounds to an aqueous system.

In conclusion, this preliminary experiment revealed that sterigmatocystin and 5,6-dimethoxysterigmatocystin disturbed ATP synthesis in mitochondria by a different manner from that of aflatoxin B_1, causing the uncoupling of oxidative phosphorylation, and that the presence of a hydroxyl group in the xanthone nucleus was important for their uncoupling activities. These mycotoxins did not induce mitochondrial swelling and did not hinder Ca^{2+}-induced swelling of mitochondria.

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