Stimulation of Aflatoxin Biosynthesis by Lipophilic Epoxides

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Epoxy fatty acids added to the culture media either with the inoculum or at the end of exponential growth phase stimulated aflatoxin production by toxigenic strains of *Aspergillus flavus* and *Aspergillus parasiticus*. This effect did not appear when the unsaturated fatty acids used for the synthesis of the epoxides and the polyhydroxyacids (which can be considered to be derived from the opening of the oxirane ring) replaced the epoxides in the culture media. No significant differences were detected in the lipid fractions (diglycerides, sterols, triglycerides, free fatty acids, sterol esters) extracted from the mycelia grown in the presence of any of the fatty acid derivatives.

INTRODUCTION

In a previous report we have described the stimulating effect of two inhibitors of fatty acid synthetase (cerulenin and tetrahydrocerulenin) on aflatoxin production (Fanelli *et al.*, 1983). This stimulating effect appeared when these compounds were added to exponential phase fungal cultures. As two analogues of cerulenin that do not inhibit fatty acid synthetase (D'Agnolo *et al.*, 1973) did not show the same effect as cerulenin, we have determined whether stimulation of aflatoxin production could be related to the epoxide structure present in cerulenin and tetrahydrocerulenin and absent in the two analogues (di- and hexahydrocerulenin). Thus we have studied the effect of methyl 9,10 epoxystearate and methyl 9,10:12,13 diepoxystearate on the biosynthesis of aflatoxins.

METHODS

Fungus culture. *Aspergillus parasiticus* (strain NRRL 2999) was normally grown in 50 ml Czapek Dox broth medium + 5 mg ZnSO₄ l⁻¹ and 1 mg Na₂MoO₄ l⁻¹ in 100 ml conical flasks at 30 °C. The inoculum was 10⁶ conidia from 15 d old culture grown on Czapek Dox agar medium. Similar experiments used strains of *Aspergillus flavus* (ATCC 22548 and CF1) as in our previous papers (Fanelli *et al.*, 1980, 1981).

Chemicals. Oleic acid, linoleic acid, oleic acid methyl ester and linoleic acid methyl ester were obtained from Sigma. The solvents for HPLC were Lichrosorb from Merck. All other chemicals were reagent grade.

Synthesis of epoxides. The synthesis of epoxides was carried out according to the methods described by Pocar (1966) with slight modifications. The methyl 9,10 epoxystearate and methyl 9,10:12,13 diepoxystearate were obtained from methyl oleate and methyl linoleate with perbenzoic acid prepared from benzylperoxide (0.21 mol) by treatment with a solution of sodium methoxide (0.22 mol).

Oleic acid methyl ester (10 mmol) was allowed to react in 150 ml diethylether with 10.5 mmol perbenzoic acid at 0 °C for 24 h with occasional shaking. The reaction mixture was washed five times with 20 ml portions 10% (w/v) NaOH, three times with distilled water and dried over anhydrous MgSO₄. The methyl 9,10 epoxystearate was recovered after evaporation of the solvent under reduced pressure in a rotary evaporator at below 30 °C and further purified on silica gel column (Si 60) using 20% diethylether/80% petroleum ether as elution solvents. The methyl 9,10:12,13 diepoxystearate was prepared as described for methyl 9,10 epoxystearate by treatment of 10 mmol methyl linoleate with 21 mmol perbenzoic acid at 0 °C for 24 h.

Synthesis of hydroxyacids. The 9,10 dihydroxystearic acid and 9,10:12,13 tetrahydroxystearic acid were obtained by performic acid oxidation followed by alkaline hydrolysis of fatty acid methyl esters (Pasi *et al.*,

1981a). Methyl oleate (1 g) was allowed to react with 15 ml formic acid plus 2 ml 30% H_2O_2 at 40 °C for 2 h with occasional shaking. The reaction mixture was diluted with 50 ml distilled water and extracted three times with 30 ml portions of peroxide-free diethylether. The extracts were pooled and the ether evaporated under reduced pressure in a rotary evaporator at below 30 °C. The product was saponified in 20 ml 3 M-NaOH at 100 °C for about 30 min, acidified at pH 2–3 with diluted H_2SO_4 and extracted as before with diethylether. The 9,10:12,13 tetrahydroxystearic acid was prepared as described for 9,10 dihydroxystearic acid by treatment of 1 g methyl linoleate with 30 ml formic acid plus 4 ml 30% H_2O_2 . Note that the opening of oxirane rings under basic or acid conditions is an $\text{S}_{\text{N}}2$ reaction, which produces *trans*-glycol. The pooled extracts containing saturated dihydroxy- and tetrahydroxystearic acids were evaporated to dryness under reduced pressure in a rotary evaporator.

Estimation of the fungal growth. Fungal growth was estimated by mycelium dry weight obtained after drying at 80 °C for 48 h. The mycelium was previously filtered on Millipore filters (pore size 0.45 μm) and washed three times in saline (NaCl 0.9%, w/v).

Analysis of lipids. The total lipids of the mycelium were extracted with $\text{CHCl}_3/\text{CH}_3\text{OH}$ (2:1, v/v) for 3 h and separated into their various components by TLC as described (Fanelli *et al.*, 1983).

Analysis of aflatoxins. The aflatoxins were extracted with $\text{CHCl}_3/\text{CH}_3\text{OH}$ (2:1, v/v) both from mycelium and culture filtrate (Fanelli *et al.*, 1981) and analysed by TLC and HPLC (Fanelli *et al.*, 1983).

Statistics. Data are presented as mean $\pm$ S.E.M. Differences from the control were evaluated by Student's test for unpaired data. Linear regression was calculated by method of least squares and their significance tested by estimating the correlation coefficient r^2 .

RESULTS AND DISCUSSION

Table 1 shows the effect of different lipid supplements added to the culture media at the end of the exponential growth phase (day 6) of the toxigenic strain of *A. parasiticus* (NRRL 2999). Among all the compounds assayed only those with epoxide ring (methyl 9,10 epoxystearate and methyl 9,10:12,13 diepoxystearate) showed an evident stimulating effect (200 times more than the control) on aflatoxin production. The stimulating effect was much more striking than that observed with cerulenin and tetrahydrocerulenin (Fanelli *et al.*, 1983). Neither the fatty acids used for the synthesis of the epoxides (oleic acid, oleic acid methyl ester, linoleic acid, linoleic acid methyl ester) nor the polyhydroxyacids (which can be considered to be derived from the opening of the oxirane ring) showed any effect on aflatoxin production.

Table 1. *Aflatoxin production, growth and total lipids extracted from mycelium by A. parasiticus at 30 °C*

Lipid supplement	Day 8			Day 10		
	Aflatoxin (μg)	Dry wt of mycelium (mg)	Total lipid (%)	Aflatoxin (μg)	Dry wt of mycelium (mg)	Total lipid (%)
Control	2.5 $\pm$ 0.7	290 $\pm$ 22	18.0 $\pm$ 5.6	2.2 $\pm$ 0.5	275 $\pm$ 18	17.7 $\pm$ 4.7
Oleic acid (1 mg ml ⁻¹)	2.0 $\pm$ 0.3	311 $\pm$ 36	19.1 $\pm$ 6.1	2.3 $\pm$ 0.4	321 $\pm$ 29	18.6 $\pm$ 5.5
Oleic acid methyl ester (1 mg ml ⁻¹)	2.4 $\pm$ 0.7	302 $\pm$ 36	18.0 $\pm$ 5.4	2.2 $\pm$ 0.6	316 $\pm$ 39	17.8 $\pm$ 4.9
Linoleic acid (0.5 mg ml ⁻¹)	2.6 $\pm$ 0.5	337 $\pm$ 41	19.4 $\pm$ 7.7	2.3 $\pm$ 0.4	327 $\pm$ 35	19.2 $\pm$ 5.2
Linoleic acid methyl ester (0.5 mg ml ⁻¹)	2.2 $\pm$ 0.3	315 $\pm$ 42	18.9 $\pm$ 4.2	2.0 $\pm$ 0.3	300 $\pm$ 27	18.8 $\pm$ 5.6
9,10 Epoxymethylstearate (1 mg ml ⁻¹)	12.4 $\pm$ 4.6*	264 $\pm$ 19	19.8 $\pm$ 6.3	233.1 $\pm$ 35.5*	387 $\pm$ 31	20.8 $\pm$ 6.1
9,10:12,13 Diepoxymethylstearate (0.5 mg ml ⁻¹)	188.2 $\pm$ 33.8*	244 $\pm$ 31	20.1 $\pm$ 6.6	261.4 $\pm$ 27.2*	374 $\pm$ 36	21.5 $\pm$ 6.7
9,10 Dihydroxystearic acid (1 mg ml ⁻¹)	3.1 $\pm$ 0.8	308 $\pm$ 40	18.5 $\pm$ 5.5	2.5 $\pm$ 0.4	296 $\pm$ 38	19.1 $\pm$ 4.5
9,10:12,13 Tetrahydroxystearic acid (0.5 mg ml ⁻¹)	2.7 $\pm$ 0.6	286 $\pm$ 37	19.1 $\pm$ 4.8	2.6 $\pm$ 0.5	312 $\pm$ 40	18.9 $\pm$ 3.9

* Statistically significant differences from the control ($P < 0.005$).

The same results were obtained when the same lipid supplements were added with the inoculum. In this case the growth of the fungus was partially inhibited by the epoxide compounds, e.g. methyl 9,10 epoxystearate and methyl 9,10:12,13 diepoxystearate inhibited growth measured as dry weight at day 6 by 40% and 50%, respectively. However, these growth inhibitory effects did not persist beyond day 8.

The lipid content of the different mycelia were unaffected by the different lipid sources used in the culture media whether or not aflatoxin production was stimulated. The lipid fractions studied were diglycerides, sterols, free fatty acids, triglycerides and sterol esters. The phospholipids phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine were all detected in all the mycelia assayed, without any significant effect of the different lipid supplements.

The fatty acid composition of the free fatty acid, triglyceride and polar lipid fractions was also examined. In all cases 95–98% of the fatty acids consisted of palmitic acid, oleic acid, linoleic acid, linolenic acid and stearic acid. The composition was unaffected by the different lipid sources whether or not they stimulated aflatoxin production.

These results confirmed our previous experiments obtained with cerulenin and tetrahydrocerulenin (Fanelli *et al.*, 1983) and their analogues although other authors have found a strict correlation between total lipid of mycelia and aflatoxin production (Shih & Marth, 1974).

Moreover, we have also reported (Fanelli *et al.*, 1981) a great increase in total lipids in mycelia grown in culture media supplemented with a higher concentration of fatty acids and triglycerides without any corresponding increase in aflatoxin production.

All the experiments reported in the present work have been repeated with other toxigenic strains of *A. flavus* (strains ATCC 22584 and CF1 isolated in our Institute). Similar results were obtained with all the strains.

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