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The Role of Cytochrome $P450_{11\alpha}$ in Detoxification of Steroids in the Filamentous Fungus *Rhizopus nigricans*

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The evidence was presented that steroid hydroxylating enzyme complex induced by substrate in the filamentous fungus *Rhizopus nigricans* (*R. nigricans*) alleviated toxic effect(s) of the steroid on fungal growth. The growth inhibition of fungal mycelium observed in steroid-containing culture(s) became much more obvious when fungal mycelia were grown in the simultaneous presence of inducing steroid and the $P450_{11\alpha}$ inhibitor metyrapone. One the other hand, in experiments where we followed the fate of radioactively labelled progesterone added to the mycelial suspension, we noticed that steroid, after being initially accumulated in the microorganism, was, after some time, released from it; the latter phenomenon was not observed if induction of 11α-hydroxylase was prevented by cycloheximide. Results of experiments presented in this communication can be regarded as the first strong indication that the biological role of $P450_{11\alpha}$ induction in *R. nigricans* is in removal of steroids which are toxic for the mycelium.

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INTRODUCTION

Transformation of steroids by prokaryotic and eukaryotic microorganisms is a widely studied and in certain cases a biotechnologically exploited mechanism [for review see ref. 1]. Data on the biological role of the transformation, whenever the latter does not represent a part of complete degradation of the steroid is, however, lacking. Although it was generally believed that such transformation of steroids taking place in certain microorganisms represented a biochemical defence mechanism, direct proof for this assumption was, to our knowledge, missing until now.

For transformation reactions introducing a hydroxyl group into a steroidal ring system in eukaryotic microorganisms the filamentous fungus *Rhizopus nigricans* was chosen as a model system. It was known from our earlier investigations that different steroids induced in this fungus a multienzyme complex which hydroxylated steroids predominantly in the 11α-position [2–5] and that this reaction required cytochrome $P450_{11\alpha}$ as terminal oxidase [6].

The aim of our investigation was to explore whether steroid hormones, added to the fungus in amounts which induced 11α-hydroxylase, were toxic for the microorganism. We designed for this purpose an experimental system which should help us to demonstrate that the toxicity of steroids depended on inactivation of the induced 11α-hydroxylase. We followed growth of *R. nigricans* by dry weight determination after growing mycelia for 18 h in the presence of either steroid alone or in the presence of the steroid added together with metyrapone as an efficient inhibitor of cytochrome $P450_{11\alpha}$. Such experiments should show whether *P*450 induced by steroids in *R. nigricans* does participate in detoxification of these compounds in the fungus or not. On the other hand, we also studied the kinetics of retention of [^{3}H]progesterone in the fungus in the absence and presence of cycloheximide as a protein synthesis inhibitor; in this way we wanted to get some insight into the fate of the hydroxylase-inducing steroid in fungal mycelia in conditions in which $P450_{11\alpha}$ was or was not induced by the steroid.

MATERIALS AND METHODS

R. nigricans ATTC 6227b was obtained from the American Type Culture Collection and was cultivated as described elsewhere [6].

The chemicals were obtained from the following companies: steroids, cycloheximide, α-naphtoflavone,

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metyrapone and β-ketoconazole (Sigma Chemical Co., St Louis, MO, U.S.A.); Bacto agar and casamino acids (Difco, U.S.A.); [1,2,6,7-^{3}H]progesterone (100 Ci/mmol) (Amersham International, England); INSTA-GEL scintillation fluid (Packard Instruments Co., Zurich, Switzerland); Silica gel plates with UV indicator (Merck, Darmstadt, Germany). All other chemicals were of analytical grade obtained either from BDH Chemicals (Dorset, England) or Sigma Chemical Co. (St Louis, MO, U.S.A.).

Growth curves

R. nigricans was grown in nutrient medium as described previously [6]. Approximately 5×10^6 spores per 100 ml medium were inoculated to each culture flask. At the time of inoculation different steroids dissolved in dimethylformamide were added to the culture medium at concentrations shown in Results. In experiments in which the effect of inhibitors of monooxygenase systems on fungal growth was studied, the inhibitors α-naphtoflavone, metyrapone and β-ketoconazole, dissolved in dimethylformamide, were added to the fungal cultures at concentrations shown in Results. Fungal mycelia were grown at 28°C in the Infors AG air thermostatic shaker. After 18 h of fungal growth, mycelia were filtered, washed with distilled water and dried until constant weight was achieved. In all experiments dry weight of three individual cultures was determined.

Enzyme induction

R. nigricans was grown as described above for 18 h, washed with distilled water and dried with filter paper. Portions of moist mycelia (4 g) were resuspended in 100 ml preheated buffer A (1 mM sodium phosphate, 0.2 mM EDTA, 0.04 mM glutathione, pH = 5.5) supplemented with 0.2% (w/v) casamino acids. Induction performed with progesterone (100 μg/ml) proceeded for 2 h at 28°C with constant shaking. In experiments where the effect of inhibitors on induction was studied the inhibitors were added to mycelial culture simultaneously with the inducer at concentrations indicated in Results.

Assay of the hydroxylation activity

2 g of moist mycelia were washed with saline [7] and incubated in 30 ml of buffer A in the presence of cycloheximide (100 μg/ml) and progesterone (100 μg/ml) as substrate. When the effect of inhibitor on fungal hydroxylation activity was studied the inhibitor was added to the assay mixtures at concentrations shown in Results. The reaction products were extracted with chloroform; after removal of the solvent by evaporation, residue was dissolved in 200 μl of chloroform. Steroids contained in 5 μl of this solution were analysed by TLC using precoated Silica gel plates with the following solvent system: chloroform–ethyl acetate–ethanol (45:45:10). Reaction products were identified using corresponding standards and percent conversion determined densitometrically (Camag TLC Scanner II).

The fate of radioactively labelled progesterone in fungal mycelia

[^{3}H]progesterone (5 μCi, 3.5×10^{-4} M) was added to 1 g mycelia resuspended in 10 ml of buffer A; the mixture was incubated for different periods of time at 28°C in a MSE shaking metabolic incubator. At time periods indicated in Results, fungal mycelia were filtered, washed and divided in two parts. In one part total cellular radioactivity was determined. The other part was used for partial subcellular fractionation; for this purpose fungal mycelia were broken in a Sorval Omni Mixer (1 g mycelia in 4 ml buffer A) and homogenate centrifuged at 15,000 *g* for 20 min. Radioactivity was determined in aliquots of resuspended sediment containing cell debris, plasma membrane fragments, nuclei and mitochondria and in aliquots of the supernatant containing cytosol and microsomal membranes. Radioactivity measurements were performed in a LKB scintillation counter using INSTA-GEL scintillation fluid.

RESULTS

Inhibition of 11α-hydroxylase in R. nigricans

*P*450 inhibitor suitable for our studies of growth inhibition of *R. nigricans* by added steroids should efficiently inhibit the 11α-hydroxylase induced by these substrates whereas mycelial growth and *P*450 induction should not be significantly affected. These requirements were met by metyrapone at a concentration of 4.5×10^{-4} M. In conditions in which the assay of hydroxylase activity was performed, 4.5×10^{-4} M metyrapone exhibited about 50% inhibition (Table 1) whereas, at this concentration, no effect on growth (Fig. 1) or hydroxylase induction (Table 2) was detected. As inhibitors of *P*450, we have also tested α-naphtoflavone and ketoconazole. The former, at a concentration of 1×10^{-4} M, not only affected fungal growth but also inhibited to some extent the induction of $P450_{11\alpha}$ and interfered with the TLC of hydroxylation products of progesterone. Although α-naphtoflavone also inhibited to some extent the activity of 11α-hydroxylase we did not use it in this investigation for

Table 1. Inhibition of 11α-hydroxylase in R. nigricans by metyrapone. 2 g of moist mycelia were incubated for 30 min at 28°C with progesterone (3×10^{-4} M) in the absence and in the presence of metyrapone. Reaction products were extracted and analysed by TLC as described in Methods

Sample	Metyrapone [M]	Inhibition of 11α-hydroxylase [relative %]
Control	0	0
A	8.9×10^{-5}	38
B	4.5×10^{-4}	52
C	2.2×10^{-3}	79

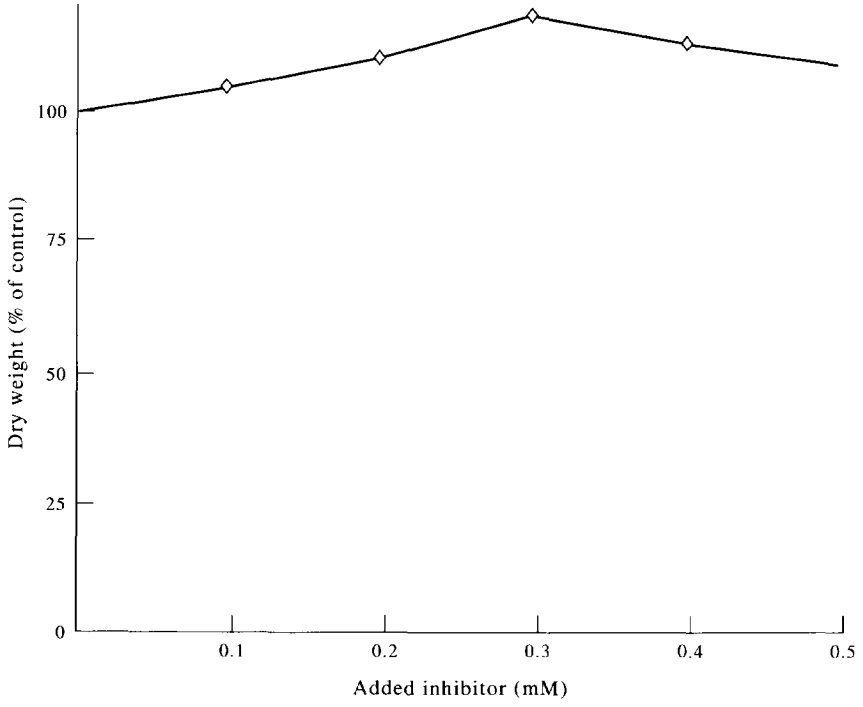

Fig. 1. Effect of metyrapone on mycelial growth of *R. nigricans*. Inhibitor was added at indicated concentrations to the inoculum of fungal spores and dry weight determined after 18 h of mycelial growth.

reasons mentioned above. Ketoconazole at 1×10^{-6} M concentration, on the other hand, turned out to be an extremely efficient inhibitor of fungal growth but had no effect on the activity of 11α-hydroxylase up to 1 mM concentration.

Effect of steroids on fungal growth

In the study of the toxic effect of steroids on filamentous fungus *R. nigricans* we have chosen three steroids which were known as good inducers and substrates of 11α-hydroxylase (deoxycorticosterone, progesterone and testosterone) [3, 5], and one which does not function either as an inducer or as a substrate of hydroxylase (estrone) [3]. We measured the effect of these steroids on fungal growth in the absence and presence of metyrapone. Results presented in Fig. 2 demonstrate that all applied steroids exerted some growth inhibition on *R. nigricans*, even in the absence of metyrapone. In this respect deoxycorticosterone was most efficient; at 5×10^{-4} M concentration of this steroid growth of the microorganism was completely arrested.

To find out whether the hydroxylation is involved in the detoxification process of steroids we measured their effect on fungal growth in the presence of metyrapone. The effect of this hydroxylase inhibitor on growth inhibition by steroids was studied at a 3×10^{-5} M concentration of steroid; at this concentration the effect of steroids on growth was negligible (Fig. 2). The results presented in Table 3 demonstrate that steroids which are metabolized by fungal 11α-hydroxylase (progesterone, testosterone and deoxycorticosterone) drastically affected fungal growth in conditions of inhibited hydroxylase, whereas estrone, which is not transformed by the fungal hydroxylase (data not shown), showed about the same inhibitory effect as in the absence of metyrapone. Results presented in Table 3 thus clearly indicate the role of 11α-hydroxylase in detoxification of steroids.

Table 2. The effect of metyrapone on induction of 11α-hydroxylase in R. nigricans. Enzyme induction was performed with 3×10^{-4} M progesterone in the absence and in the presence of metyrapone. At the end of induction mycelia were washed and tested for hydroxylation activity in the presence of cycloheximide as described in Methods

Sample	Metyrapone [M]	Hydroxylation activity [relative %]
Control	0	100
A	8.9×10^{-5}	124
B	4.5×10^{-4}	98
C	2.2×10^{-3}	80

The fate of [^{3}H]progesterone in fungal mycelia

To get some explanation for the detoxification effect of 11α-hydroxylase induced in the presence of steroids which act as substrates for this enzyme in *R. nigricans*, the fate of [^{3}H]progesterone was followed in fungal mycelia in the absence and presence of cycloheximide. For this purpose the radioactivity was assayed, at different periods of time after addition of radioactively labelled progesterone to the mycelial culture, in total homogenate on one side, and in supernatant and sediment of the centrifuged homogenate (as described in Methods) on the other side. The results of these experiments, as presented in Fig. 3, can explain the role

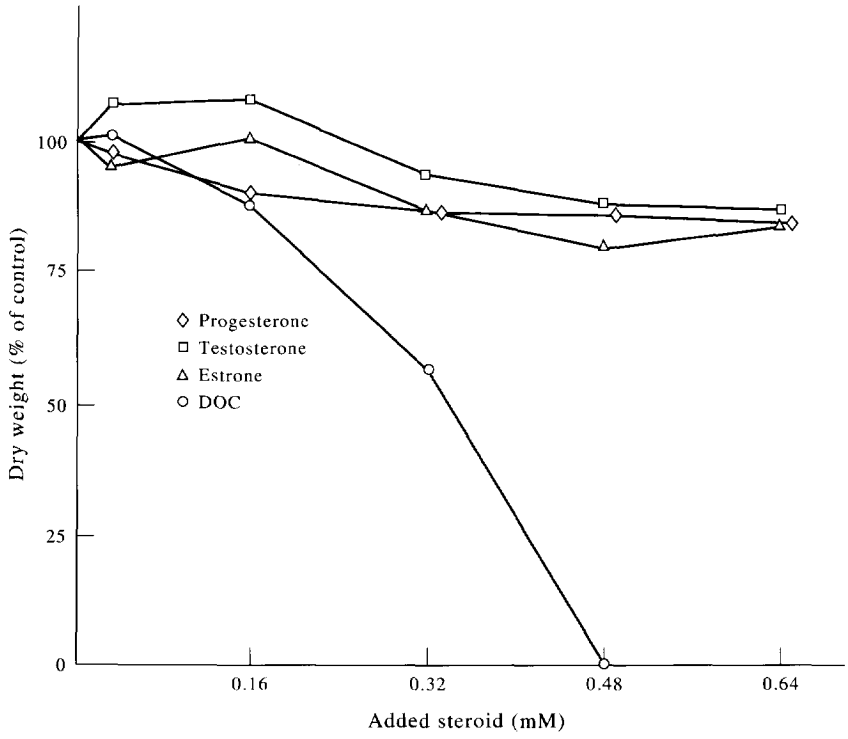

Fig. 2. Effect of different steroids on mycelial growth in the absence of 11α-hydroxylase inhibitors. Steroids were added at indicated concentrations during inoculation of fungal spores and the dry weight determined after 18 h of mycelial growth. DOC, deoxycorticosterone.

of the introduction of a hydroxyl group into the steroidal ring system. The steroid which was accumulating in all structures of the fungal mycelia was removed from it only when the hydroxylase was induced whereas if the fungus was treated with progesterone in the presence of cycloheximide, the steroid remained bound to mycelial structures.

DISCUSSION

Until now there was not data available on *P*450 inhibitors in *R. nigricans*. We tested several substances known from the literature as effective inhibitors of *P*450 enzyme systems. Metyrapone was known as an efficient inhibitor of $P450_{11\beta}$ since it acts on the protein as well as on the heme moiety of the enzyme [8]; this inhibitor was also found to be very effective in inhibition of 11α-hydroxylation of steroids in *R. nigricans*. Our results are, therefore, in agreement with what is known for the action of this inhibitor on $P450_{11\beta}$. Metyrapone, by inhibiting the action of $P450_{11\alpha}$ without affecting its induction, enabled us to show the growth inhibition effect of steroids which otherwise would not be detected because of the action of the induced 11α-hydroxylase.

Table 3. The influence of 11α-hydroxylase inhibition by metyrapone on toxic effect of steroids on fungal growth. Spores of R. nigricans were inoculated in the presence of 10 μg/ml of respective steroid and 4.5 × 10^{-4} M metyrapone. Dry weight was determined after 18 h of mycelial growth as described in Methods. Progesterone (P), testosterone (T), deoxycorticosterone (DOC), estrone (E_1)

Growth conditions	Dry weight (rel. %)	
	− metyrapone [mean ± SD]	+ metyrapone [mean ± SD]
No steroid	100	100
P	90.2 ± 8	68.7 ± 8
T	95.0 ± 4	80.4 ± 2
DOC	90.8 ± 4	65.2 ± 3
E_1	90.8 ± 4	95.8 ± 6

The observation that α-naphtoflavone inhibited the induction of $P450_{11\alpha}$ and that estrone, which did not turn out to be an inducer, was also not toxic to the mycelium, seemed interesting for further work connected with studies on mechanism of induction of 11α-hydroxylase by steroids. If steroids act during induction via specific receptor proteins present either on the plasma membrane [9] or/and in the fungal cytosol it could be expected that α-naphtoflavone and esterone would act as antagonists of 11α-hydroxylase inducing steroids.

Results on the fate of [^{3}H]progesterone in fungal mycelia are also in good agreement with experimental data presented above where we showed that the growth inhibition was much higher in conditions in which the hydroxylase was inhibited by metyrapone. The introduction of the hydroxyl group most probably rendered the hydrophobic steroid molecule more water soluble and the steroid herewith lost to some extent its affinity for different hydrophobic fungal constituents. If steroids indeed interfered with membraneous function

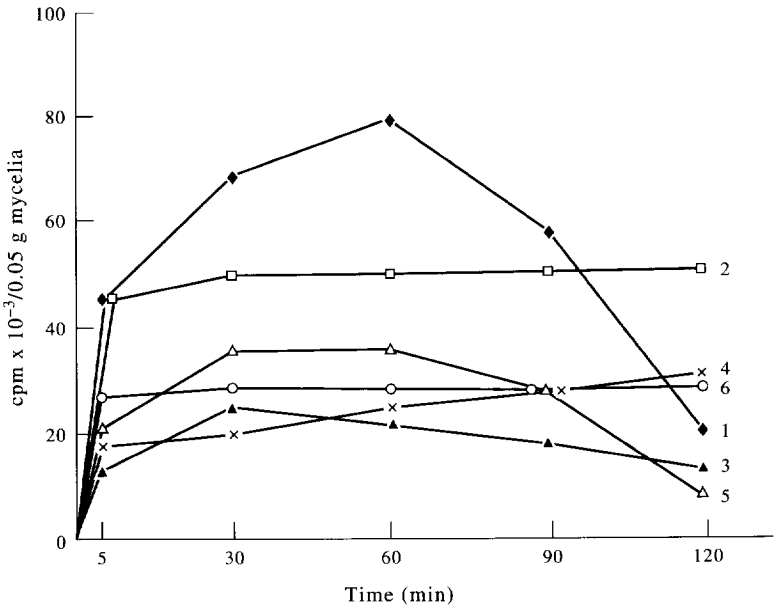

Fig. 3. Time dependent [^{3}H]progesterone retention in mycelia and some subcellular fractions of *R. nigricans*. [^{3}H]progesterone was added after 42 h of mycelial growth and radioactivity was measured at indicated times in samples prepared as described in Methods. (1) Fungal mycelia, (2) mycelia in presence of cycloheximide, (3) 15,000 *g* sediment, (4) 15,000 *g* sediment in presence of cycloheximide, (5) cytosol + microsomes, (6) cytosol + microsomes in presence of cycloheximide.

by binding to different nonpolar structures, this effect was eliminated by removal of steroid from the fungus.

In conclusion we can say that experimental results presented in this report show clearly that $P450_{11\alpha}$ is induced in *R. nigricans* as a defence mechanism. The purpose of the 11α-hydroxylation is, therefore, inactivation of steroids which exert toxic effects on the fungus. The fungal $P450_{11\alpha}$ could be compared according to its function with cytochromes *P*450 which in the liver of higher organisms introduce hydroxyl groups into steroid molecules at many different positions in order to inactivate these compounds.

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