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Transformation of 3-hydroxy-steroids by *Fusarium moniliforme* 7α-hydroxylase

Anne-Cécile Cotillon [1], Robert Morfin*

Laboratoire de Biotechnologie, Conservatoire National des Arts et Métiers, 2 rue Conté, 75003, Paris, France

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Abstract

Transformation of physiologically important 3-hydroxy-steroids by the DHEA-induced 7α-hydroxylase of *F. moniliforme* was investigated. Whereas DHEA was almost totally 7α-hydroxylated, PREG, EPIA and ESTR were only partially converted into their 7α-hydroxylated derivatives because hydroxylation at other undetermined positions as well as reduction of ketone at C17 or C20 into hydroxyl also occurred. Cholesterol was not transformed by the enzyme. Kinetic parameters of the 7α-hydroxylation for these substrates were determined and confirmed that DHEA was the best substrate of the 7α-hydroxylase. Inhibition studies of DHEA 7α-hydroxylation by the other 3-hydroxy-steroids were also carried out and proved that DHEA, PREG, EPIA and ESTR shared the same active site of the enzyme. Induction effects of these steroids were compared, and DHEA appeared to be the best inducer of the 7α-hydroxylase of *F. moniliforme*.

1. Introduction

Irreversible 7α-hydroxylation of native 3β-hydroxy-steroids (particularly DHEA and PREG) by rat [1–4], mouse [4–6] and human [7–10] tissues has been investigated since 1966. Because of irreversibility of the P450-catalyzed hydroxylation process and their lack of hormonal activity [11,12], 7α-hydroxylated metabolites of sex hormone precursors were regarded as physiologically inactive molecules, and for a long time 7α-hydroxylation was solely considered as a mechanism of regulation of active steroid hormone concentration [13–15]. However, recent studies [16,17] indicated that 7α-hydroxylated metabolites of 3β-hydroxy-steroids increase immune response in mouse and may have antiglucocorticoid potencies. These findings implied that 7α-hydroxy-steroids might play a key role in the regulation of glucocorticoid action and the immune process. In other work [18], the 7α-hydroxylation of 27-hydroxy-cholesterol was regarded as another pathway alternate to that of cholesterol 7α-hydroxylation for the production of primary bile acids. At present, further studies of the 7α-hydroxylation mechanism are hindered because none of the 7α-hydroxylated derivatives of 3-hydroxy-steroids is commercially available.

Fungi have been frequently used for performing and studying specific steroid hydroxylations [19–22] and, like *Rhizopus nigricans* for the synthesis of glucocorticoids, they can even be industrially exploited. We have previously reported [23] that the fungal strain *F. moniliforme* contained a microsomal P450 enzyme system that could be induced by DHEA, thus allowing production of very large quantities of 7α-hydroxy-DHEA. In the present study, we investigated the conversion of other 3β-hydroxylated steroids (PREG, EPIA and cholesterol) and of a 3-hydroxy-steroid (ESTR) by the DHEA-induced 7α-hydroxylase of *F. moniliforme*, as

Abbreviations: DHEA, dehydroepiandrosterone; PREG, pregnenolone; EPIA, epiandrosterone (3β-hydroxy-5α-androstan-17-one); ESTR, estrone; PBS, phosphate buffered saline; PMSF, phenylmethylsulfonylfluoride; BSTFA, bis-(trimethylsilyl) trifluoroacetamide; TMS, trimethylsilyl; SEM, standard error of the mean.

* Corresponding author. Tel.: +33-140272572; fax: +33-140272380.

E-mail address: morfin@cnam.fr (R. Morfin)

[1] On leave from ENGREF (Ecole Nationale du Génie Rural des Eaux et des Forêts), Paris, France.

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well as the induction of the fungal 7α-hydroxylase by these steroids.

2. Materials and methods

2.1. Chemicals

[4-^{14}C]-DHEA (51 mCi/mmol), [4-^{14}C]-ESTR (52.5 mCi/mmol) and [4-^{14}C]-cholesterol (51.3 mCi/mmol) were purchased from NEN (Du Pont de Nemours, France). [20-^{14}C]-PREG (55 mCi/mmol) was produced (custom synthesis CFQ 6416) by Amersham (Cardiff, UK). [4-^{14}C]-EPIA (58.3 mCi/mmol) was prepared at the laboratory from [4-^{14}C]-5α-dihydrotestosterone (NEN) by means of enzymatic conversion. Briefly, [4-^{14}C]-5α-dihydrotestosterone (150 nmol) was incubated with *E. coli* HB-101 transformed with pPT-1 plasmid containing the (3-17)β-hydroxy-steroid dehydrogenase gene of *P. testosteroni* [24] provided by Dr Abalain (Medical School, Brest, France) in NADH-fortified 100 mM tris buffer (pH 9.0) at 30°C for 20 min. Ethyl acetate-extracted [4-^{14}C]-EPIA (41.9%) was separated from [4-^{14}C]-5α-androstane-3,17-dione (26%), [4-^{14}C]-5α-androstane-3β,17β-diol (5.4%) and non-transformed [4-^{14}C]-5α-dihydrotestosterone (21.3%) by TLC, and its radiochemical purity was periodically checked by crystallization to constant specific activity of a carrier-diluted portion [25]. Non-radioactive reference DHEA, EPIA and ESTR were purchased from Sigma-Aldrich (St Louis, MO, USA). Reference PREG was a gift from Roussel-Uclaf (Romainville, France). 7α-Hydroxy-DHEA, 7β-hydroxy-DHEA, 7α-hydroxy-PREG, 7β-hydroxy-PREG, 7α-hydroxy-EPIA, and 7β-hydroxy-EPIA were obtained or prepared as previously reported [3,26]. 7α-Hydroxy-ESTR and 7β-hydroxy-ESTR were gifts from Schering AG (Berlin, Germany). Solvents, salts and chromatographic supplies were purchased from Merck (Darmstadt, Germany). Sucrose, D-(+)-glucose, yeast extract, beef extract powder, PBS, EDTA, NADPH, pepstatin, leupeptin, PMSF and cycloheximide were from Sigma-Aldrich. BSTFA was obtained from Supelco (Bellefonte, PA, USA).

2.2. Organism

The strain of *F. moniliforme*, provided by the Department of Microbiology of the University of Brest (Prof. D. Thouvenot), was cultivated as already described [23]. After 2 days of fungal growth, mycelia were recovered on sintered glass filters and weighed prior to the induction procedure.

2.3. Induction of mycelia

Portions of mycelia (4 g) were resuspended in fresh nutrient medium (100 ml) and unlabeled steroid (DHEA unless otherwise stated), dissolved in 0.2 ml ethanol, was added for a final concentration of 0.3 mM. Conditions of induction (temperature, shaking) were identical to those used for fungal growth. After 18 h, mycelia were filtered, washed with cold PBS for 2 h and suspended in 1 ml of PBS containing EDTA (1 mM), sucrose (0.5 M) and glycerol (30%), and pH was adjusted to 7.4. Resulting mycelia suspensions were frozen in liquid nitrogen and stored at −80°C prior to microsome preparation.

2.4. Preparation of microsomal fraction

Mycelia were pulverized in liquid nitrogen with a SPEX 6700 freezer mill purchased from Bioblock Scientific (Illkirch, France) and operated at the highest impact frequency for 5 min. The powder obtained was collected in ice-cold PBS containing EDTA (1 mM), glycerol (20%), pepstatin (2 μM), leupeptin (2 μM) and PMSF (0.1 mM), pH 7.4. Homogenization was carried out at 2°C with 10 up and down strokes in a glass-teflon homogenizer set at 600 rpm. Nuclei and cell debris were pelleted by centrifugation of the homogenates at 800 × g for 15 min. Mitochondria were removed by centrifugation at 12,000 × g for 20 min and recovered supernatant was then centrifuged at 105,000 × g for 60 min to yield the microsomal pellet. Microsomes were suspended in PBS containing EDTA (1 mM), glycerol (20%), sucrose (0.5 M), pepstatin (2 μM), leupeptin (2 μM) and PMSF (0.1 mM), pH 7.4, and stored at −80°C prior to protein measurement and incubations.

2.5. Incubations

The ethanol solution of ^{14}C-labeled steroid (0.5 nmol) was dried under vacuum in a Speed-vac concentrator (Savant Instrument Corp., Hicksville, NJ, USA) at the bottom of 10-ml glass tubes. For kinetic studies, serial isotopic dilutions of the radioactive steroid were made prior to drying. Incubations were carried out in a total volume of 1 ml in phosphate buffer (Na_2HPO_4/KH_2PO_4 67 mM, pH 7.4) containing EDTA (1 mM) as previously described [23]. For kinetic and inhibition studies, the quantity of microsomal protein was 0.1 mg and incubation time was 10 min. These parameters were selected after preliminary assays showed a linear increase in 7α-hydroxy-metabolite production during a 0–15 min period. Incubations were stopped by addition of 1 ml of acetone and steroids were extracted, separated, quantified and identified.

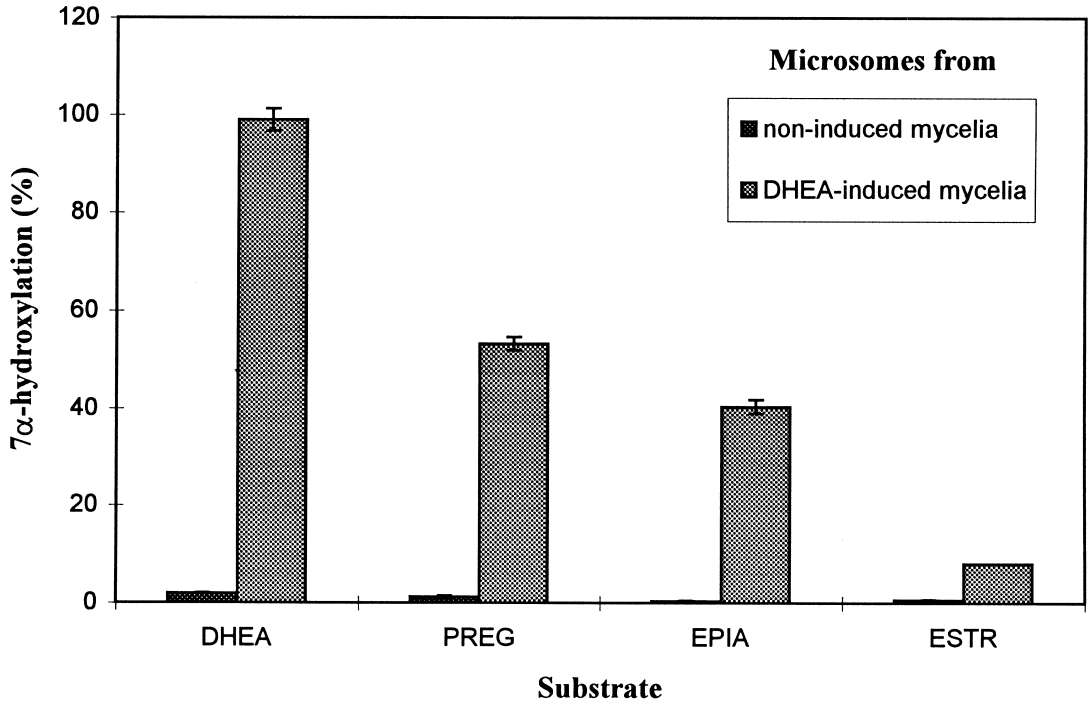

Fig. 1. 7α-Hydroxylation of [4-^{14}C]-DHEA, [20-^{14}C]-PREG, [4-^{14}C]-EPIA and [4-^{14}C]-ESTR by microsomes prepared from both non-induced and DHEA-induced mycelia of *F. moniliforme*. ^{14}C-labeled steroids (0.5 μM) were incubated with microsomes (0.5 mg protein) in NADPH-supplemented phosphate buffer (total volume 1 ml) at 27°C for 30 min. Means ± SEM resulted from three experiments.

2.6. Extraction and separation of radiosteroids

Water phases with acetone were extracted 3 times with 2 ml ethyl acetate. Recoveries in the organic phase were in the 95–99% range. Extracts were dried under vacuum, dissolved in 0.1 ml ethyl acetate, and applied to silica-gel F_{254} thin-layer plates for TLC. Reference steroids were applied on separate lanes, and the plates were developed once in ethyl acetate. With this system, steroids were separated at R_f 0.76, 0.82, 0.73, and 0.88 for DHEA, PREG, EPIA and ESTR, respectively. The 7-hydroxylated derivatives of these steroids were also separated, at $R_f = 0.25$ for 7α-hydroxy-DHEA, $R_f = 0.28$ for 7α-hydroxy-PREG, $R_f = 0.32$ for 7α-hydroxy-EPIA, $R_f = 0.71$ for 7α-hydroxy-ESTR, $R_f = 0.40$ for 7β-hydroxy-DHEA, $R_f = 0.53$ for 7β-hydroxy-PREG, $R_f = 0.36$ for 7β-hydroxy-EPIA and $R_f = 0.69$ for 7β-hydroxy-ESTR.

2.7. Quantification of radiosteroids

After TLC, the relative amounts of ^{14}C-labeled substrates and metabolites were measured by scanning of the thin-layer plates with a Multitrace-Master LB-2832 (Berthold Analytical Instruments, Nashua, IL, USA).

2.8. Identification of metabolites

A preliminary identification of labeled steroids was performed by comparison of their R_f with those of authentic steroids. A second and more reliable identification was carried out by GC/MS analysis with a Hewlett-Packard HP 5890 series II gas chromatograph coupled with an HP 5989A mass spectrometer. TMS ether derivatives of reference and unidentified steroids were prepared by reacting the dried steroids with 0.1 ml BSTFA at 60°C for 40 min and injected in the column. Programmation of oven temperature as well as characteristics of ionization chamber have been already described [23].

2.9. Other analytical procedures

Protein concentrations were measured with bicinchoninic acid reagent (Kit from Pierce) at 562 nm with bovine serum albumin (Sigma-Aldrich) used for calibration [27]. Linear regressions, means and SEM were computed from data resulting from experiments repeated 3 to 4 times.

3. Results

3.1. 7-hydroxylation of DHEA, PREG, EPIA and ESTR by microsomes of F. moniliforme

[4-^{14}C]-DHEA, [20-^{14}C]-PREG, [4-^{14}C]-EPIA, [4-^{14}C]-ESTR and [4-^{14}C]-cholesterol were incubated

Table 1
Retention times and relative intensities of characteristic ion fragments obtained after gas chromatography/mass spectrometry analysis of trimethylsilyl ether (TMS) derivatives of authentic 7α-hydroxy-PREG, 7α-hydroxy-EPIA, 7α-hydroxy-ESTR, and of the metabolites of PREG, EPIA and ESTR with the same R_f as reference 7α-hydroxy-metabolites

Steroids (TMS derivatives)	Retention time (min)	Percent relative abundance of detected ions					
		M^+	M^+-15	M^+-90	M^+-105	M^+-2 × 90	$C_6H_{13}OSi^+$ (m/z 129)
Reference 7α-hydroxy-PREG	19.53	nd[a]	nd	100	5.34	nd	13.5
Eluted 7α-hydroxy-PREG	19.47	nd	nd	100	2.4	nd	3.57
Reference 7α-hydroxy-EPIA	17.86	nd	64.5	82.3	15.1	100	96.6
Eluted 7α-hydroxy-EPIA	17.75	nd	36.1	100	12.4	52.7	28.7
Reference 7α-hydroxy-ESTR	19.31	4.16	1.3	100	4.26	nd	1.65
Eluted 7α-hydroxy-ESTR	19.38	12.9	7.51	100	11.2	nd	7.41

[a] nd: not detected.

with microsomal fractions from either non-induced or DHEA-induced mycelia of *F. moniliforme* for 30 min. TLC of extracts showed that each substrate tested, except cholesterol, was converted into its 7α-hydroxylated derivative, but other metabolites were also produced. Yields of 7α-hydroxylation were very low at the constitutive level (< 2%), but they were greatly increased after induction of fungal 7α-hydroxylase by DHEA (Fig. 1). For the concentration tested, conversion of DHEA into its 7α-hydroxylated derivative was almost total (approximately 98%) after 30 min of incubation with microsomes prepared from induced *F. moniliforme*, and it was approximately 1.8, 2.4 and 12.2 times higher than that obtained with PREG, EPIA and ESTR, respectively.

3.2. Identification of 7-hydroxylated metabolites of PREG, EPIA and ESTR

The di-TMS derivatives of authentic 7-hydroxy-steroids and of steroids extracted from incubation buffer were prepared for GC/MS analysis. Characteristic ion fragment patterns and retention times of authentic 7α-hydroxy-PREG, EPIA and ESTR were obtained in extracts from PREG, EPIA and ESTR incubations respectively (Table 1), thus showing 7α-hydroxylation of PREG, EPIA and ESTR by *F. moniliforme*. Analysis of ion fragment patterns obtained at other retention times indicated that the other metabolites of PREG, EPIA and ESTR were dihydroxylated products different from 7β-hydroxy-steroids, and also triols resulting from hydroxylation and reduction of the ketone at C17 or C20. Most of these metabolites could not be identified due to lack of reference steroids. 6α-Hydroxy-EPIA was not detected in extracts from EPIA incubation. 7α-Hydroxy-estradiol was identified as one of the triols detected in extracts obtained from ESTR incubation. Neither DHEA inducer nor 7α-hydroxy-DHEA was detected in extracts from PREG, EPIA and ESTR incubations. Thus, microsomes were

Table 2
Proportions and R_f of metabolites recovered in extracts from DHEA, PREG, EPIA and ESTR incubations with DHEA-induced microsomes of *F. moniliforme*. ^{14}C-labeled steroids (0.5 μM) were incubated with fungal microsomes (0.5 mg protein) in NADPH-supplemented phosphate buffer at 27°C for 30 min. Clues for identity of unidentified metabolites were based on spectra obtained from GC/MS analysis

Steroid substrate	7α-hydroxylated metabolite		Additional unidentified metabolites		
	R_f	%	R_f	%	Clues
DHEA	0.25	98	0.14	Traces	Triol?
PREG	0.28	53	0.15	12	Triol?
			0.39	4.5	Hydroxylated metabolite?
			0.47	8.8	Hydroxylated metabolite?
EPIA	0.32	40	0.24	Traces	Triol?
			0.50	45	Hydroxylated metabolite?
			0.62	Traces	Hydroxylated metabolite?
ESTR	0.71	8	0.63	7	7α-hydroxy-estradiol + Hydroxylated metabolite?
			0.8	Traces	Triol? + hydroxylated metabolite?

Table 3
Kinetic parameters of the 7α-hydroxylation of DHEA, PREG, EPIA and ESTR by microsomes derived from DHEA-induced mycelia of *F. moniliforme*. ^{14}C-labeled steroids (0.5 nmol) diluted with increasing concentrations of unlabeled steroid were incubated with microsomes (0.1 mg protein) in phosphate buffer supplemented with NADPH (0.5 mg) at 27°C for 10 min. Total volume was 1 ml. K_M and V_{max} values (means ± SEM) were obtained from Lineweaver-Burk plots using data from three experiments

Substrate	K_M (μM)	V_{max} (pmol/min/mg protein)
DHEA	1.18 ± 0.035	909 ± 27
PREG	1.89 ± 0.208	263 ± 18.4
EPIA	1.73 ± 0.15	294 ± 25.6
ESTR	5.38 ± 0.575	256 ± 27.4

depleted of DHEA inducer and of its 7α-hydroxylated metabolite during the preparation of microsomal fractions. Results are summarized in Table 2.

3.3. Kinetics of DHEA, PREG, EPIA and ESTR 7α-hydroxylation

Microsomes prepared from DHEA-induced mycelia were incubated with diluted ^{14}C-labeled substrate. Computation of 7α-hydroxylation yields was obtained from experiments repeated 3 times. The Lineweaver-Burk plot gave apparent K_M and V_{max} values for DHEA, PREG, EPIA and ESTR 7α-hydroxylation (Table 3). The K_M value measured for DHEA was approximately 1.4 times smaller than those obtained for PREG and EPIA, and about 4 times smaller than K_M of ESTR 7α-hydroxylation. V_{max} values of PREG, EPIA and ESTR 7α-hydroxylation, ranging from 256 to 294 pmol/min/mg protein, were approximately 3–3.5 times smaller than that obtained for DHEA 7α-hydroxylation.

3.4. Inhibition of DHEA 7α-hydroxylation by PREG, EPIA and ESTR

Since PREG, EPIA and ESTR were 7α-hydroxylated by microsomes derived from DHEA-induced mycelia of *F. moniliforme*, it was interesting to determine the type of inhibition they exert on DHEA 7α-hydroxylation. Microsomes from DHEA-induced fungi were incubated with diluted [4-^{14}C]-DHEA and inhibitor. For each steroid tested as inhibitor, use of the Lineweaver-Burk plot with data from three experiments yielded unchanged V_{max} and increased K_M, providing evidence for a competitive inhibition of DHEA 7α-hydroxylation by these steroids. Computations using Dixon plots confirmed this conclusion and gave K_i values (Table 4). K_i obtained with PREG and EPIA were not statistically different ($P > 0.1$), whereas value calculated for ESTR was approximately 13.5 times larger.

Table 4
K_i values of inhibition by PREG, EPIA and ESTR of DHEA 7α-hydroxylation by microsomes prepared from DHEA-induced *F. moniliforme*. ^{14}C-labeled DHEA (0.5 μM) diluted with unlabeled DHEA (1, 2 and 4 μM total) was incubated with steroid inhibitor (0–8 μM) and microsomes from DHEA-induced fungi (0.1 mg protein) in phosphate buffer supplemented with NADPH (0.5 mg) at 27°C for 10 min. Total volume was 1 ml. K_i (means ± SEM) were obtained from Dixon plots using data from three experiments

Inhibitor	K_i (μM)
PREG	1.05 ± 0.222
EPIA	1.18 ± 0.728
ESTR	15 ± 3.62

3.5. Influence of steroid used as inducer

Except for traces of other metabolites, 7α-hydroxy-DHEA was the only metabolite produced after incubation of DHEA substrate with microsomes prepared from fungi induced with DHEA, and the yield of 7α-hydroxy-DHEA production was 98% in the conditions tested. In contrast, incubation of PREG, EPIA and ESTR led to production of other detectable metabolites in addition to 7α-hydroxylated derivatives, and the yields of 7α-hydroxylation did not exceed 50% in the same conditions. Thus, it was interesting to test whether 7α-hydroxylation of PREG, EPIA, and/or ESTR could be increased when a steroid other than DHEA was used as inducer of 7α-hydroxylase. To test this hypothesis, mycelia of *F. moniliforme* were exposed to DHEA, PREG, EPIA or ESTR prior to microsome preparation, and incubations of each ^{14}C-labeled steroid were carried out with each microsomal fraction. For each steroid substrate tested, 7α-hydroxylating activity was maximum in microsomes prepared from DHEA-induced mycelia (Fig. 2). It was approximately 1.8, 3.9 and 12.5 times higher than in microsomes prepared from mycelia induced by PREG, EPIA and ESTR, respectively.

4. Discussion

In a previous report [23] we demonstrated that *F. moniliforme* carried out DHEA 7α-hydroxylation, and that the responsible P450-containing enzyme system was inducible by DHEA. We proved here that the DHEA-induced 7α-hydroxylase of *F. moniliforme* could convert other 3-hydroxylated steroids, namely PREG, EPIA and ESTR, into their 7α-hydroxylated metabolites. However, this could not be generalized, because cholesterol was not transformed. Identification of the 7α-hydroxy-steroids produced was based on TLC and GC/MS analysis.

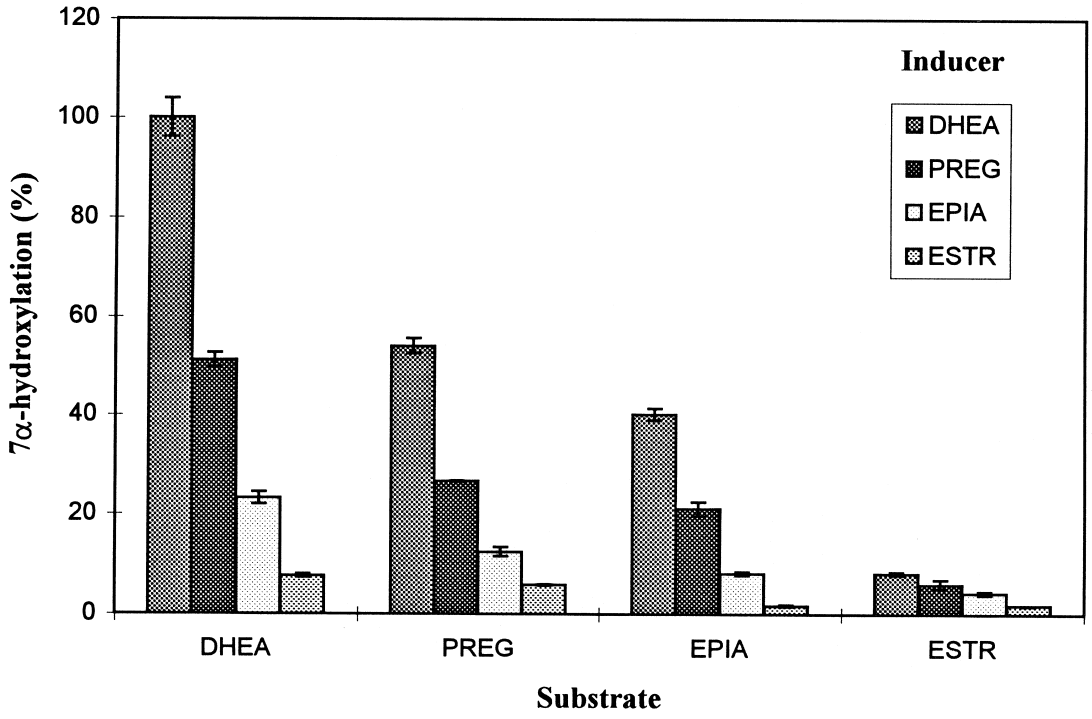

Fig. 2. 7α-Hydroxylation of [4-^{14}C]-DHEA, [20-^{14}C]-PREG, [4-^{14}C]-EPIA and [4-^{14}C]-ESTR (0.5 nmol) by microsomes (0.5 mg protein) prepared from mycelia of *F. moniliforme* induced by PREG, EPIA or ESTR for 18 h. Incubations were carried out in phosphate buffer supplemented with 0.5 mg NADPH at 27°C for 30 min. Means ± SEM resulted from three experiments.

7α-Hydroxylation yields of PREG, EPIA and ESTR by the DHEA-induced fungal 7α-hydroxylase were found to be approximately 1.8, 2.4 and 12.2 times lower than that of DHEA, respectively. Whereas 7α-hydroxy-DHEA was almost the sole metabolite formed, PREG, EPIA and ESTR were not only hydroxylated at the 7α-position, but hydroxylations at other non-determined position positions also occurred, as well as reduction of the ketone at C17 or C20. These additional metabolites could not be identified due to lack of steroid references. Determination and comparison of kinetic parameters of 7α-hydroxylation of DHEA, PREG, EPIA and ESTR indicated a strong affinity of DHEA-induced 7α-hydroxylase for DHEA, as well as a high V_{max}; in contrast, and in addition to decreased V_{max}, a lower affinity of this enzyme was found for other steroids tested, particularly ESTR. Thus, K_M and V_{max} values were in agreement with 7α-hydroxylation yields obtained for each substrate. Inhibition studies of DHEA 7α-hydroxylation by PREG, EPIA and ESTR revealed competitive inhibition types, thus proving that PREG, EPIA and ESTR share the same active site of the enzyme with DHEA. Use of Dixon plots gave a high K_i value for the inhibition exerted by ESTR and about 13.5-times lower K_i values for PREG and EPIA inhibitions. These results were in agreement with K_M and 7α-hydroxylation yields obtained and indicated that, whereas PREG and EPIA are relatively 'good' substrates for DHEA-induced 7α-hydroxylase of *F. moniliforme*, ESTR is a rather 'bad' substrate and DHEA an 'excellent' substrate. Cholesterol was no substrate at all.

Comparison of substrate structures could give some clues on the mechanism of steroid-enzyme interaction: PREG differs from DHEA by the presence of an oxidized 2-carbon side-chain at C17, while cholesterol has no oxygen in a long side-chain. EPIA and ESTR have the same oxo group at C17 as DHEA, but both differ from DHEA by the absence of the C5—C6 double bond, and in addition ESTR has a phenolic ring A. Thus we could assume that a steroid substrate might interact with the DHEA-induced 7α-hydroxylase by its C17 or C20 oxygen, and that the presence of both the C5—C6 double bond and the 3β-hydroxyl might facilitate positioning of the substrate in the active site because of sterical or electronic reasons. The differences observed in hydroxylation yields might also be caused by the difference of solubility of the steroids tested. Others [28,29] have shown that the ability of a steroid to reach an enzyme active site depends, at least partially, on the solubility-dependent steroid concentration in the phospholipid layer of microsome membranes. Another group [30] found that polarity of side-chain at C17 was one important factor determining the yield of 11α-hydroxylation of 3-keto-4-ene steroids by *Rhizopus nigricans*.

We may hypothesize that a sole unspecific enzyme,

rather than several regiospecific enzymes, was responsible for all hydroxylations of investigated steroid substrates, because other work proved that one hydroxylase can catalyse steroid hydroxylations at different positions. It was demonstrated [31] that one cytochrome P450 catalyzes both 11β-and 14α-hydroxylation of 11-deoxycortisol in *Curvularia lunata*. The 11α-, 6β- and 7β-hydroxylations of substrates by *Rhizopus nigricans* were also shown to be most probably due to a unique enzyme with three attachment sites [30]. Our present investigation excluded 7β-hydroxylation from the possible additional activity of 7α-hydroxylase. Since fungi of the *F.* species were reported to carry out 15α-hydroxylations of some steroids, including EPIA [32], unidentified hydroxylations of the steroids tested might be 15α-hydroxylations. But in other fungal strains, 7α-hydroxylation was associated with 15β-hydroxylation [33] or with 14α-hydroxylation [34]. Furthermore, if we consider the rotation of a steroid along its long axis, 7α and 11β positions become equivalent for enzyme-substrate complexes [35]. Thus, some of the major undetermined metabolites of PREG, EPIA and ESTR might be 11β-hydroxy-derivatives. Contrary to other substrates, DHEA was mostly hydroxylated at the 7α position. This may be due to a tight positioning of DHEA in the enzyme active site preventing rotation and leading to exclusive exposition of C7 to the P450 heme. Formation of triols indicates that, in addition to hydroxylation reactions, 17-oxo-steroids were converted into 17-hydroxy-steroids. This transformation was catalyzed either by the same DHEA-induced enzyme, or by another enzyme, an oxido-reductase present in the microsomal fraction of *F. moniliforme*. Since this oxido-reductase activity was not detected in microsomes derived from non-induced fungi, three explanations are made possible: either this oxido-reductase was induced by DHEA, or the substrate had to be hydroxylated prior to being reduced by the oxido-reductase, or the DHEA-induced 7α-hydroxylase itself carried out the reduction of ketone at C17 or C20 into alcohol. Such a C17 oxido-reductase activity was also observed with steroid biotransformations by *Nectria haematococca* [36] and *Rhodotorula mucilaginosa* [37].

Since the DHEA-induced enzyme of *F. moniliforme* was very specific for 7α-hydroxylation of DHEA and carried out only partial 7α-hydroxylation of other substrates tested, it was logical to test whether exposure of mycelia to PREG, EPIA or ESTR prior to microsome preparation could increase the 7α-hydroxylation yields of these steroids through induction of another more specific 7α-hydroxylase. Results indicated that several 3-hydroxy-steroids may induce 7α-hydroxylating activities in microsomes of *F. moniliforme*, but among the steroids tested, 7α-hydroxylated derivative production was maximum in microsomes prepared from DHEA-exposed mycelia and induction abilities of steroids tested were strongly correlated with their 7α-hydroxylation yields: DHEA was the best inducer of *F. moniliforme* 7α-hydroxylase, whereas ESTR was a very bad inducer of the enzyme. Nevertheless, this result cannot be generalized as a rule, because other investigators demonstrated that, although it was a very good substrate for *Mucor piriformis* 14α-hydroxylase, 16-dehydroprogesterone was not a good inducer of the enzyme [38]. In addition, deoxycorticosterone and testosterone were demonstrated to be better inducers of *Rhizopus nigricans* 11α-hydroxylase than progesterone, but were poorer substrates than this steroid [39]. Induction effect on *Cochliobolus lunatus* 11β-hydroxylase was shown to strongly depend on the structure of the steroid inducer and to be especially influenced by C17 substituents [40].

DHEA and PREG are termed as neurosteroids because of their production in brain [3]. Studies of their metabolism proved their 7α-hydroxylation [4], but conclusions remained speculative on their possible neuroendocrine effects. Additional work proved that 7α-hydroxylated metabolites of DHEA and PREG are very potent stimulators of the immune response in mouse [16], and studies in progress indicate that 7α-hydroxy-EPIA may have the same potency. Since chemical synthesis of these physiologically important molecules yields small amounts and requires costly purification steps, the results presented here should prove to be very useful for production of these native steroids, needed for further investigations on their biological actions.

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