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Biotransformation of 17β-hydroxy-11β-(4-dimethylaminophenyl)17α-1-propynyl-estra-4,9-dien-3-one (RU486) in rat hepatoma variants

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Abstract—Metabolism of the synthetic steroid 17β-hydroxy-11β-(4-dimethylaminophenyl)17α-1-propynyl-estra-4,9-dien-3-one (RU486) occurs in the dedifferentiated S-H56-125 variant of Reuber hepatoma. Considering that rat liver cytochrome P450 (P450) monooxygenases are engaged in different oxidative steps of the metabolism of RU486, the influence of several prototype P450 inducers was investigated. The data obtained by treating H56 and S-H56-125 hepatoma cells with different P450 inducers (dexamethasone (DEX), benzanthracene, phenobarbital) or with a specific P450 inhibitor, troleandomycin, led us to conclude that CYP3A is involved in the hydroxylation of RU486. This form is induced by DEX independently of the availability of the canonical glucocorticoid receptor.

17β-Hydroxy-11β-(4-dimethylaminophenyl)17α-1-propynyl-estra-4,9-dien-3-one (RU486*) has numerous clinical applications including birth control, treatment of Cushing's syndrome and typical cancers [1]. The biological effectiveness of RU486 is greatly influenced by its metabolism. Seven metabolites exist in rat bile or plasma from either mono- or didemethylation of the 11β-4-dimethyl-aminophenyl ring, or hydroxylation of the 17α-propynyl group [2]. The cytochrome P450 (P450) monooxygenases [3] are involved in the biotransformation of RU486 in rat liver [4]. Since different families of P450 are probably engaged in the degradation process, investigations have been shifted to a simplified system of cultured hepatoma cells. A major impediment to cellular and molecular studies of liver-specific proteins including P450s derives from the absence of suitable culture systems for *in vitro* studies. Cultured cell lines like most hepatomas become dedifferentiated, a characteristic which is frequently associated with the loss of responsiveness to P450 inducers [5]. The differentiated Reuber rat hepatoma Fao clone, which is sensitive to several inducers, especially phenobarbital (PB), benzanthracene (BA) and dexamethasone (DEX), hardly metabolizes RU486 [6, 7]. The establishment of a few DEX-resistant Reuber hepatoma clones which actively metabolize RU486 has enlarged the scope for study [8, 9]. Two cell lines have been chosen for their properties: H56 cells containing glucocorticoid receptors (GRs) whose growth is sensitive to DEX and also S-H56-125 cell variants whose growth is DEX-resistant since their altered GR does not bind DEX [10]. Interestingly, DEX-sensitive and -resistant variants (in relation to growth) differ in their ability to degrade RU486. P450s are involved in the metabolism process of the steroid since reduced metabolism of RU486 is observed in the presence of aminoglutethimide, an inhibitor of liver P450 [9].

Materials and Methods

Chemicals. DEX phosphate, RU486 and [6-7-^{3}H]RU486 (sp. act. 55 Ci/mmol) were provided by the Roussel-Uclaf Co. (Romainville, France). PB was from Special (Pams, France), BA from Fluka (Buchs, Switzerland) and troleandomycin (TAO) from the Sigma Chemical Co. (Poole, U.K.). All other chemicals were of the highest grade of purity obtained from different sources.

Cell lines and culture conditions. The glucocorticoid-sensitive and/or -resistant cell lines and the culture conditions have been described previously [8, 11]. The cells were grown in Ham's F-12 medium (Gibco, Uxbridge, U.K.) supplemented with 5% fetal calf serum (Gibco) at 37° in a humidified atmosphere of 5% CO_2 and air. Cells were harvested in a 0.05% trypsin–0.02% EDTA solution. The H56 cell clone is a spontaneously dedifferentiated derivative of the H4IIEC3 rat hepatoma cell line [12, 13] that fails to express liver-specific functions characteristic of the differentiated hepatomas [14]. The growth of H56 cells is inhibited by glucocorticosteroids like DEX [8, 11]. For isolation of DEX-resistant variants, H56 cells were treated with 400 μg/mL ethyl methansulfonate for 20 hr. The survivors (about 30%) were grown in the presence of increasing concentrations of DEX for 10 months. By subcloning the DEX-resistant H56 cells in a medium containing 2 μM DEX, the subclone S-H56-125 was obtained [11].

In vivo *metabolism of RU486 in hepatoma cells.* When assessing the metabolism of chemicals, it is important to standardize the growth culture conditions, since the activities of drug-metabolizing enzymes change considerably during the growth cycle [15]. Thus, the metabolism of RU486 was studied in cells cultured for 5 days after they had been seeded into culture flasks (2×10^6 cells per 75 cm^2 dish). The final density was the same for the two cell lines (about 12×10^6 cells) and the culture was not confluent. The hepatoma cells were incubated with 10 nM tritiated steroid for different periods of time. Thereafter, the plates were rinsed three times with saline and the cells removed in 600 μL saline with a rubber bulb. The unconverted steroid and its metabolites were extracted and separated by TLC [16]. Moderate degradation of the steroid consecutive to slight decomposition of RU486 in contact with the culture medium is subtracted (5–10%).

Results

Considering that rat liver P450 monooxygenases are engaged in different oxidative steps of steroid degradation, the influence of different prototype P450 inducers was studied. Both inducers, either PB for CYP2B,† CYP2C and for CYP3A to a lesser extent [17], or BA, inducing CYP1A [18], did not modify the rate of RU486 metabolism (Table 1). As in untreated cells, after 6 hr contact, 26% of the radioactivity was found in the peak corresponding to intact RU486. The other 74% was mainly found in the two peaks corresponding to the 11β-monodemethylated derivative RU42633 and to material which no longer migrates (M5). The 11β-didemethylated derivative

* Abbreviations: BA, benzanthracene; P450, cytochrome P450; DEX, dexamethasone; GR, glucocorticoid receptor; PB, phenobarbital; TAO, troleandomycin; RU486, 17β-hydroxy-11β-(4-dimethylaminophenyl)17α-1-propynyl-estra-4,9-dien-3-one.

† P450 nomenclature proposed by Nelson *et al.* [3].

Table 1. Metabolism of RU486 in S-H56-125 and H56 cells untreated (C) or treated with DEX (1 μM), BA (1 μM) or PB (2 mM)

		S-H56-125				H56			
		C–PB–BA		DEX		C–PB–BA		DEX	
	R_f	3 hr	6 hr	3 hr	6 hr	3 hr	6 hr	3 hr	6 hr
RU486	0.69	60 ± 3	31 ± 3	39 ± 6	14 ± 3	96 ± 4	94 ± 4	85 ± 3	70 ± 3
RU42633	0.60	17 ± 2	24 ± 3	22 ± 3	27 ± 3	3 ± 2	5 ± 2	5 ± 2	9 ± 3
RU42698	0.26	5 ± 2	6 ± 2	9 ± 2	12 ± 2	UD	UD	4 ± 2	9 ± 2
RU42848	0.16	8 ± 2	9 ± 2	8 ± 2	10 ± 2	UD	UD	2 ± 1	2 ± 1
M5	0.00	10 ± 3	30 ± 3	22 ± 3	37 ± 3	1 ± 1	1 ± 1	4 ± 2	10 ± 3

The cells were incubated with [^{3}H]RU486 for 3 or 6 hr. The labeled steroids (RU486 and its metabolites) were chromatographed; their position (R_f) and relative amount in the samples analysed were evaluated by integrating the surface of the peak after scanning (the background was subtracted).

The results represent the percentage of each steroid in the sample (UD, undetectable). Values are the means of four experiments ± SD.

RU42848 and the 17α-hydroxylated derivative RU42698, characterized in rat liver by Deraedt *et al.* [2], hardly appeared. An 18 hr pretreatment of the cells with DEX (1 μM), an inducer of CYP3A but also of CYP2B and CYP2C [19, 20], increased metabolism. However, only 12% of the labeled steroid remained intact after 6 hr contact. This enhancement was paralleled by a significant increase in the 17α-hydroxylated derivative. The ratio of M5 increased slightly, while the percentage of demethylated derivatives did not change significantly. The effect of DEX on the metabolism of RU486 lasted at least 6 hr after removal of DEX from the culture medium. Thus, the presence of the inducer during or immediately before incubation with the antihormone is not a prerequisite to enhancing the metabolism. In the H56 parental cell line, the metabolism of RU486 occurred only after treatment with DEX while PBA and BA were without any effects (Table 1). Quantitative analysis of the metabolites following DEX treatment was difficult due to the low amounts present. However, the presence of RU42672 was obvious. When S-H56-125 and H56 cells were treated simultaneously with DEX and PB or DEX and BA, the rate of RU486 metabolism was the same as in cells treated with DEX alone (data not shown).

Since the glucocorticoid-inducible CYP3A catalyses the hydroxylation of endogenous steroids and also participates in the demethylation process [21, 22]. H56 and S-H56-125 cells were incubated with a selective inhibitor of CYP3A, TAO [23], after pretreatment (18 hr) with DEX. The medium was replaced by DEX-free medium before adding TAO (1 μM) and labeled RU486. After a 6 hr incubation, the DEX-induced metabolism of RU486 was blocked in both cell lines (Fig. 1). Moreover, TAO strongly diminished the metabolism of RU486 in untreated S-H56-125 cells. The drastic inhibition of RU486 metabolism in the presence of TAO was not due to the toxicity of the compound since the viability and growth of the cells did not change under TAO treatment (data not shown).

Previous studies show that RU486 does not induce P450 but antagonizes the inductive effects of DEX and PB on different P450 forms [7]. These findings lead to examination of whether RU486 acts on its own metabolism. Cells were first treated with RU486 (1 μM) for 18 hr and incubated thereafter with the labeled antihormone for different periods of time. Treatment with RU486 did not change the rate of RU486 metabolism (Fig. 2). In contrast, by treating the cells simultaneously with RU486 (1 μM) and DEX (1 μM) for 18 hr, the DEX-induced metabolism was diminished in both H56 and S-H56-125 cells.

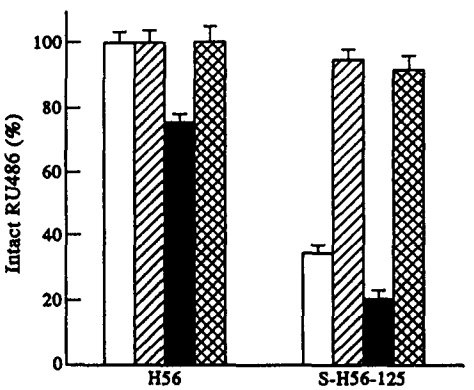

Fig. 1. Influence of TAO on the metabolism of RU486 in H56 and S-H56-125 hepatoma cells. Cultured cells were untreated (□, ▨) or treated with DEX for 18 hr (■, ▩), before being incubated with [^{3}H]RU486 for 6 hr in the presence (▨, ▩) or absence (□, ■) of TAO. The relative amount of labeled steroids (RU486 and its metabolites) in the sample was analysed. The values are the means of four experiments (SD: bars).

Discussion

The present study led to the following conclusions: (i) CYP3A, which has been shown previously to be induced by DEX [19, 20], is probably engaged in the hydroxylation steps of RU486 and also participates in the demethylation process of the antihormone; (ii) CYP3A may be expressed constitutively in S-H56-125 but not in H56 cells and it may be upregulated in both cell types by DEX. This is independent of the GR since one cell type has functional classical receptors whereas the other cell type does not [10]. The antiglucocorticoid RU486 inhibits P450 induction by DEX whereas other antiglucocorticoids (progesterone and pregnenolone 16α-carbonitrile) have been shown to be unable to block induction by DEX [19]. Therefore, it can be assumed that the inducibility of CYP3A is not mediated by the GR. It is worth noting that an unusual, non-classical GR-mediated process for induction of the CYP3A gene by DEX has been proposed [20, 21, 24]. Interestingly, PB which is claimed to induce CYP3A [17],

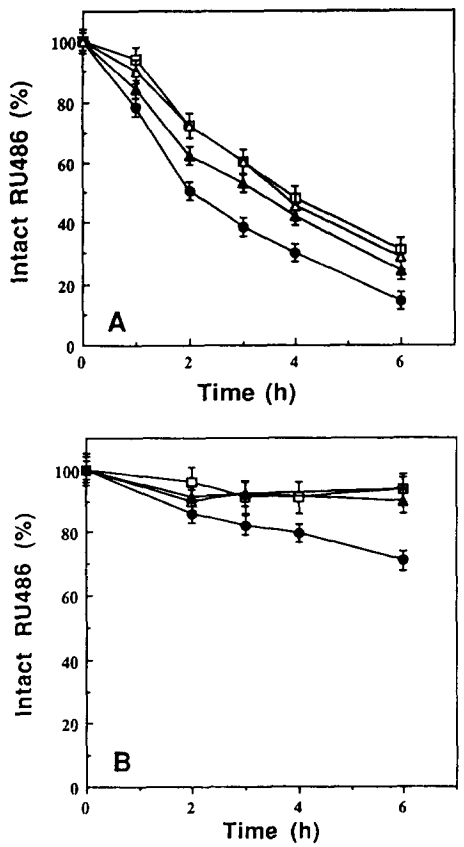

Fig. 2. Influence of RU486 on its own degradation in S-H56-125 (A) and H56 cells (B) treated with DEX. The cells were untreated (□), treated during 18 hr with RU486 (△), DEX (●), or a combination of RU486 and DEX (▲). Thereafter, the cells were incubated with [^{3}H]RU486 for different periods of time. The relative amount of labeled steroids (RU486 and its metabolites) in the sample was analysed. The values are the means of four experiments (SD: bars).

did not exert any effect when it was given to the hepatoma variants either alone or in combination with DEX. The presently available model system should make it possible to elucidate the molecular mechanisms involved in CYP3A gene control.

CYP1A1, which is highly inducible by BA in both the differentiated and dedifferentiated hepatomas [6], seems not to be involved in the metabolism of RU486 because treatments of H56 or S-H56-125 cells with BA were without any effect on the metabolism of the antihormone. This corroborates earlier findings where the treatment of rats with 3-methylcholanthrene, the other prototype inducer of CYP1A, does not increase the metabolic activity of liver microsomes toward RU486 [3].

All the contributions to RU486 metabolism performed either on rat liver or on different hepatoma cells (Fao, H56, S-H56-125 cells) suggest that CYP1A does not seem to be engaged in the metabolism of RU486. CYP2B and 2C contribute markedly to the demethylation process of the molecule whereas CYP3A is responsible mainly for the hydroxylation steps.

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