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REDUCTIVE METABOLISM OF METYRAPONE BY A QUERCITRIN-SENSITIVE KETONE REDUCTASE IN MOUSE LIVER CYTOSOL

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Abstract—Mouse liver cytosol catalyses the reduction of metyrapone to the corresponding alcohol metabolite metyrapol. The enzyme involved was characterized as a NADPH-dependent carbonyl reductase which is strongly inhibited by the plant flavonoid quercitrin but which shows no sensitivity to phenobarbital. Thus, by inhibitor subdivision of carbonyl reductases the metyrapone reductase in mouse liver cytosol has to be classified as a ketone reductase rather than an aldehyde reductase, as it was shown previously for the analogous enzyme in mouse liver microsomes based on the same pattern of inhibitor classification. Moreover, immunological comparison of the metyrapone reductases from the two subcellular fractions reveal no common antigenic determinants indicating the structural difference between these enzymes. In conclusion, metyrapone undergoes reductive biotransformation mediated by two clearly distinct carbonyl reductases located in different subcellular compartments of mouse liver cells. Considering the convenient and sensitive HPLC-method for direct metyrapol determination, metyrapone may serve as a useful tool in the investigation of these enzymes, although their physiological roles remain to be determined.

Metyrapone (2-methyl-1,2-bis-(3-pyridyl)-1-propanone, Su 4885) is an extensively studied inhibitor of the adrenal mitochondrial 11 β-hydroxylating system [1, 2]. In this respect it is used clinically as a diagnostic agent for the determination of ACTH secretion from the anterior pituitary gland, as a diuretic in some cases of resistant oedema, and in treatment of Cushing's syndrome [3, 4].

Metyrapone also inhibits the microsomal monooxygenase [5] and gained importance as a diagnostic tool in characterizing different forms of cytochrome P450 isozymes [6].

Although it interacts with cytochrome P450 like a type II substrate [5] hydroxylation, which leads to metyrapone *N*-oxides [7], is not the main metabolic pathway of metyrapone. Pharmacokinetic studies have shown that it is almost completely reduced at its ketone function to the corresponding alcohol metabolite metyrapol (Su 5236) [8] before being eliminated as unchanged metyrapol and, to the largest extent, as metyrapol *N*-oxides [9].

Information on the enzyme system mediating the reductive biotransformation of metyrapone in the microsomal fraction were first reported by Sprunt *et al.* [10]. More detailed studies by Kahl [11] revealed that only 6% of the total homogenate activity could be found in rat liver cytosol, whereas a mean value of 65% was obtained in the microsomal fraction. In previous investigations we isolated and purified the metyrapone reducing enzyme from mouse liver microsomes and characterized it as a 34 kDa carbonyl reductase which was classified by inhibitor sensitivity to phenobarbital as an aldehyde reductase capable of using NADH or NADPH as cosubstrate [12].

The present communication describes results of studies on the subcellular distribution of metyrapone reducing activity in the mouse liver as well as the cosubstrate dependency and the sensitivity of the cytosolic metyrapone reductase to diagnostic inhibitors of carbonyl reductases. Moreover, using antibodies raised against the microsomal mouse liver metyrapone reductase we checked the cytosolic protein for immunological homology to the microsomal enzyme performing the immunoblot technique.

METHODS AND MATERIALS

Animals. Female NMRI mice (25–30 g) were used for the experiments.

Chemicals. Metyrapone was purchased from Fluka AG (Buchs, Switzerland). Enzymatic tests were performed using NADH, $NADP^+$, NADPH, G-6-P, G-6-P-DH from Boehringer Mannheim (Mannheim, F.R.G.). For HPLC acetonitrile of HPLC-grade from E. Merck (Darmstadt, F.R.G.) was used. For inhibitor studies quercitrin and dicoumarol were purchased from Roth (Karlsruhe, F.R.G.), phenobarbital and pyrazole from Fluka (Neu-Ulm, F.R.G.) and 5α-dihydrotestosterone from Aldrich Chemie (Steinheim, F.R.G.). All other chemicals used in the experiments were reagent grade and were obtained from commercial suppliers.

Preparation of subcellular fractions. After perfusing the livers with an ice cold isotonic solution of KCl they were homogenized in 4 volumes of 20 mM Tris–HCl buffer, pH 7.4, containing 250 mM sucrose and 1 mM EDTA using a glass–Teflon Potter-Elvehjem homogenizer. The homogenate was centrifuged at 600 *g* for 10 min to sediment the nuclei and cell debris. The resulting supernatant was centrifuged at 10,000 *g* to sediment mitochondria.

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The supernatant at this stage was centrifuged at 170,000 *g* for 1 hr to sediment microsomes, finally giving a clear cytoplasmic supernatant. The sediment pellets at each stage were washed twice with 10 mL of the homogenizing buffer and then suspended in 1 mL of the same buffer. Dialysis was carried out overnight against 2000 vol. of a 50 mM sodium phosphate buffer, pH 7.4.

Purification of the microsomal metyrapone reductase: The purification of the microsomal metyrapone reductase for immunization was carried out by preparative SDS–PAGE as described by Irrgang *et al.* [13] except that for electroelution of proteins the apparatus of Gerton *et al.* [14] was used.

Immunization and preparation of antisera: 1 mg of the enzyme preparation was dissolved in 62.5 mM Tris–HCl, 0.1% (w/v) SDS, 0.5 mM EDTA, pH 6.8 and emulsified with 1 vol. complete Freund's adjuvant. For immunization, a female rabbit was injected subcutaneously (0.9 mL emulsion containing about 0.3 mg protein (2 ×) or 0.2 mg protein (2 ×) per injection) four times on the following days: 1./3./5./36., the latter being the booster.

Immunoblot. Electrophoretically separated proteins were transferred to nitro cellulose sheets [15]. Antisera against the microsomal metyrapone reductase were diluted 1:1,000 and incubated with protein saturated nitrocellulose sheets. Antigen–antibody complexes were detected by peroxidase conjugated secondary antibodies specific for rabbit IgG (dilution 1:1,000). These complexes were visualized by the peroxidase reaction (chloronaphthol method) as shown by Hawks *et al.* [16].

Enzyme assay. Assay of metyrapone reductase was performed by preincubating 100 μL of enzyme solution in 50 mM sodium phosphate buffer, pH 7.4. For inhibitor studies 10 μL of the respective inhibitor were added to a final concentration of 1 mM. Inhibitors which were not sufficiently soluble in buffer were dissolved in ethanol or 0.04 M NaOH. Control velocities were determined in the presence of appropriate quantities of the solvents. After the preincubation period of 3 min the reaction was started by adding 10 μL of metyrapone (final concentration 4.6 mM, which was found to be saturating) and 10 μL of the respective cosubstrate (final concentrations: NADH 3.2 mM; NADPH 3.2 mM; NADPH-regenerating system: $NADP^+$ 0.8 mM, G-6-P 6 mM, G-6-P-DH 0.35 units, MgCl 3 mM) to a final volume of 150 μL. The reduction was stopped after 30 min by mixing 50 μL of the reaction sample with 150 μL of ice cold acetonitrile. The samples were centrifuged for 6 min at 8,000 *g* in the cold and 20 μL of the supernatant served for the determination of metyrapone and metyrapol by HPLC-analysis.

Determination of metyrapone and metyrapol by HPLC. A HPLC-system combined of a 6000 A solvent pump and a model 440 absorbance detector (Waters Assoc., Milford, MA, U.S.A.) and an injector block (Rheodyne, 7125) served for chromatographical analysis. Samples of 20 μL each prepared as described above were analysed on a C_{18}-column (Serva octadecyl-Si 100 polyol, 4.6 × 25 mm). The eluent was 30% acetonitrile in a 50 mM phosphate buffer, pH 7.4, at a flow rate of 1 mL/min. The absorbance was recorded at 254 nm.

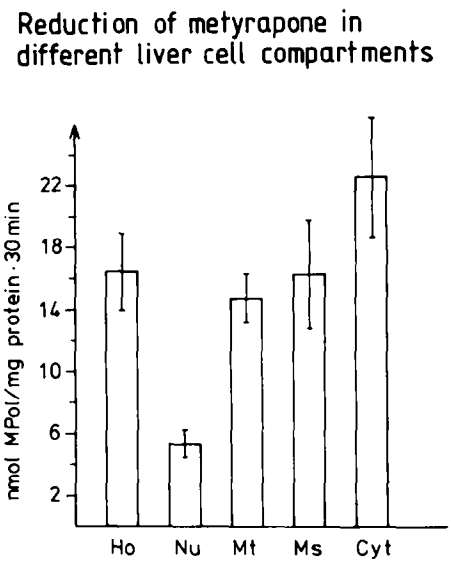

Fig. 1. Reduction of metyrapone in different cell compartments of mouse liver. The subcellular fractions were obtained by differential centrifugation (Ho = homogenate, Nu = nuclei, Mt = mitochondria, Ms = microsomes, Cyt = cytoplasm) (cf. Methods). The specific activity was assayed in the standard reaction mixture containing 50 mM sodium phosphate buffer, pH 7.4, 4.6 mM metyrapone and a NADPH-regenerating system. Activity is expressed as nmol metyrapol formed in 30 min per mg of protein.

Analytical techniques. Protein concentration was determined by the method of Lowry *et al.* [17] using bovine serum albumin as standard. NADPH-cytochrome *c*-reductase activity as marker enzyme for the endoplasmic reticulum was measured in each subcellular fraction according to the method of Williams and Kamin [18] using cytochrome *c* from horse heart assuming an extinction coefficient of 19.1 $cm^{-1}mM^{-1}$.

RESULTS AND DISCUSSION

Experiments on the subcellular distribution of metyrapone reducing activity reveal metyrapol formation in all compartments of liver cells obtained by differential centrifugation, i.e. the nucleic, mitochondrial, microsomal and cytoplasmic fractions (cf. Fig. 1). Whereas in livers of rats metyrapone reduction has been classically considered to occur primarily in the microsomal fraction [10, 11] the reductase activity in the cytosolic fraction in livers of mice is even higher than in the respective microsomes (cf. Fig. 1). Nevertheless, significant metyrapone reductase activity is also associated with the nucleic and mitochondrial fractions. This might be due to the relatively high contamination of these two fractions with endoplasmic reticulum, as determined by measuring cytochrome P450 reductase, the typical microsomal marker enzyme and

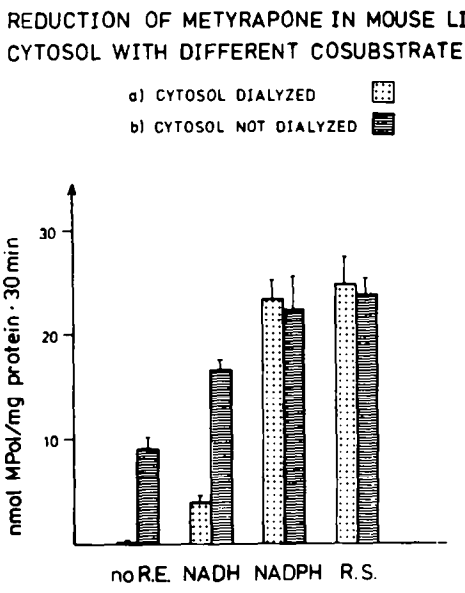

Fig. 2. Reduction of metyrapone in mouse liver cytosol with different cosubstrates. The specific activity was assayed in the standard reaction mixture containing 50 mM sodium phosphate buffer, pH 7.4, 4.6 mM metyrapone and either no reducing equivalents (no R.E.), 3.2 mM NADH, 3.2 mM NADPH or a NADPH-regenerating system (R.S.), respectively. Activity is expressed as nmol metyrapol formed in 30 min per mg of protein.

Table 1. Effects of inhibitors on metyrapone reduction in mouse liver cytosol

Inhibitor	Concentration (mM)	Residual enzyme activity (%)*
None	—	100
Pyrazole	1	103
Phenobarbital	1	99
5α-DHT†	1	96
Dicoumarol	1	93
Indomethacin	1	57
Quercitrin	1	46

* The enzyme activity was assayed in the standard reaction mixture containing 50 mM sodium phosphate buffer, pH 7.4, 4.6 mM metyrapone, a NADPH-regenerating system and inhibitor concentrations of 1 mM, respectively. The percentages are calculated from uninhibited control experiments.

† 5α-DHT = 5α-dihydrotestosterone.

which probably arises during organelle preparation. However, in the 170000 *g* supernatant almost no cytochrome P450 reductase was detectable, giving evidence for the absence of any contaminating endoplasmic reticulum in this fraction.

One of the typical features of carbonyl reductases is their preference for NADPH as a cosubstrate [19]. Since the microsomal metyrapone reductase can use either NADPH or NADH as cosubstrate [12], we tested the same electron donors in the cytosolic fraction. Although significant metyrapone reduction occurs with NADH, the preferred cosubstrate is either NADPH or an NADPH-regenerating system (cf. Fig. 2). The strong preference of the enzyme activity for NADPH rather than for NADH becomes more distinct after removal of the naturally occurring reducing equivalents by dialysis (cf. Fig. 2).

Thus, the cytosolic metyrapone reducing enzyme shows a universal characteristic of carbonyl reductases with regard to its specificity for NADPH as electron donor.

Inhibitor studies (cf. Table 1) reveal that 5α-dihydrotestosterone, the strongest inhibitor of the microsomal metyrapone reductase and pyrazole, the potent inhibitor of alcohol dehydrogenase, do not influence metyrapol formation at all. Dicoumarol, the specific inhibitor of NADP(H):quinone oxidoreductase (EC 1.6.99.2. DT-diaphorase) decreased it weakly to 93% and indomethacin to 57%, respectively.

Of relevance is the sensitivity of the enzyme to the diagnostic inhibitors phenobarbital and quercitrin, which are used to subdivide the carbonyl reductases into phenobarbital-sensitive aldehyde reductases and quercitrin-sensitive ketone reductases [20]: whereas phenobarbital does not affect metyrapone reduction, quercitrin decreases it to about 46%. Thus, based on inhibitor classification of carbonyl reductases the cytosolic metyrapone reductase is a ketone reductase rather than an aldehyde reductase, as it was shown for the microsomal enzyme [12].

For estimating immunological homologies between the metyrapone reducing enzymes of the microsomal and cytosolic fractions the immunoblot technique was applied. Microsomal and cytosolic protein was separated by SDS gel electrophoresis and blotted to nitrocellulose sheets. Antigen–antibody complexes were identified by the peroxidase reaction of the secondary conjugated antibody specific for rabbit IgG. Polyclonal antibodies raised in rabbits against the homologous microsomal metyrapone reductase specifically crossreacted with one single protein band of the microsomal fraction corresponding to the homologous enzyme in the 34 kDa molecular weight region (cf. Fig. 3). The association of the immunostained band to Coomassie-stained protein pattern in the polyacrylamide gel is based on the relative position of the crossreacting protein band to that of the original antigen. However, no crossreaction occurred with any protein of the cytosolic fraction, indicating the absence of common antigenic determinants (cf. Fig. 3). Moreover, immunoinhibition experiments with the antibodies against the microsomal enzyme reveal a decrease in specific activity of 21% in microsomes (data not shown) but no inhibition of the cytosolic reductase. From these findings it becomes obvious that the microsomal and cytosolic enzymes are not structurally related, despite their common specificity to metyrapone as a substrate for carbonyl reduction.

In conclusion, the diagnostic inhibitor of oxidative drug metabolism metyrapone is reduced to the corresponding alcohol in mouse liver cytosol by a NADPH-dependent carbonyl reductase which is sensitive to the flavonoid quercitrin and insensitive

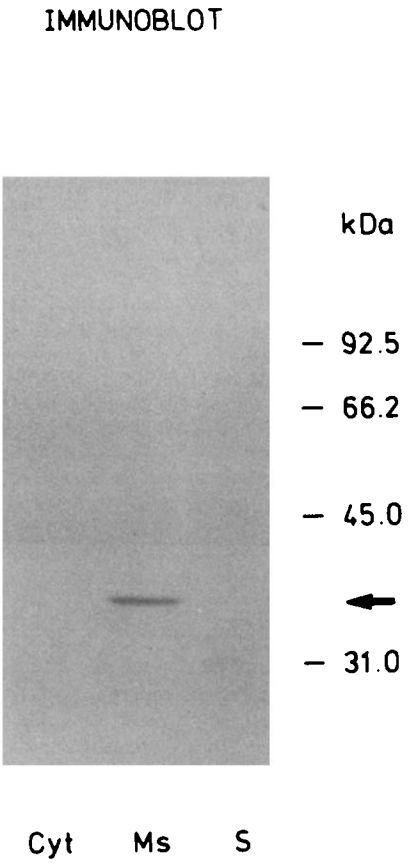

Fig. 3. Immunoblot of the cytosolic and microsomal fractions after SDS polyacrylamide gel electrophoresis. Polyclonal antibodies raised in rabbits against the microsomal metyrapone reductase were incubated as primary antibody. Antigen–antibody complexes were identified by the peroxidase reaction of the secondary conjugated antibody specific for rabbit IgG. The arrow indicates the homologous antigen of the microsomal fraction in the 34 kDa molecular weight region. (Cyt = cytoplasm, Ms = microsomes, S = molecular weight standard.)

to phenobarbital. By inhibitor subdivision of carbonyl reductases [20] this enzyme therefore can be classified as a ketone reductase.

In previous investigations it was shown that the analogous metyrapone reducing enzyme from mouse liver microsomes has a contrary sensitivity to the above mentioned diagnostic inhibitors and consequently was classified as an aldehyde reductase [12]. The two enzymes do not have any common antigenic determinants indicating their structural difference, although they show similar specificity for metyrapone as substrate. Thus, under identical conditions the ketone metyrapone in one and the same reaction serves as a substrate for two clearly distinct enzymes which, in addition, are localized in different subcellular compartments of the same tissue. However, their physiological roles remain to be determined.

In contrast to extensive studies on other metabolic routes of xenobiotic biotransformation, ketone and aldehyde reductases up to now have obtained less attention. Primarily, our studies contribute to the elucidation of the reductive metabolism of metyrapone. Furthermore, metyrapone may be used as a useful tool in investigating enzymes involved in the reductive biotransformation of carbonyl compounds, since it was shown to be mainly reduced *in vivo* to the respective alcohol, rather than being oxidized at the nitrogens of the two pyridine rings [8, 9]. Moreover, the method of HPLC analysis for direct determination of the product metyrapol is clearly advantageous in comparison to the indirect spectrophotometric recording of pyridine nucleotide oxidation at 340 nm usually employed.

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