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Cytochrome P450 1A1 and 4A activities in isolated rat spleen lymphocytes

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In this study the activity of Cytochrome P 450 (CYP) enzymes in isolated spleen lymphocytes of Wistar rats was estimated. 7-Ethoxyresorufin-*O*-deethylase was analysed to determine the activity of CYP 1A1/2. The activity was inreased 2.2-fold by pretreating the animals with 3-methylcholanthrene, and 1.3-fold after the addition of the mitogen concanavalin A to the lymphocyte culture. Pretreatment of the rats with phenobarbitone did not enhance the basal enzyme activity. To estimate reductive activities the azoreduction of 4-(*N,N*-dimethyl-amino)azobenzene was analysed. Again detectable turnovers were found which could be increased 4-fold after the addition of concanavalin A. Azoreductase activity could also be increased 2-fold after clofibrate was added to the lymphocyte cultures, and was decreased by adding metyrapone so that it was concluded that azoreduction may be partly due to the CYP 4A-family.

1. Introduction

The use of animal and human cells for studying the biotransformation of xenobiotics is of increasing importance. Primary cell cultures as well as cell lines with appropriate biochemical abilities for metabolizing drugs make it possible to clarify the metabolic spectrum of active substances. Lymphocytes contain detectable activities of the microsomal monooxygenase system cytochrome P450 (CYP) and are therefore important for the metabolism of xenobiotics [1–3]. In this study the presence and inducibility of CYP 1A1/2 was examined in isolated spleen lymphocytes of untreated rats as well as of rats pretreated with enzyme inducing agents.

7-Ethoxyresorufin (ER) is a specific substrate of CYP 1A1 and 2. These are induced by planar molecules like the polycyclic aromatic hydrocarbon 3-methylcholanthrene (3-MC) [4]. CYP 1A1 and 2 can be quantified specifically by the 7-ER assay, whose highly fluorescent product resorufin can easily be measured [5].

To detect reductive activities azoreduction of 4-(*N,N*-dimethyl-amino)azobenzene (DAB) was used. DAB azoreduction is induced by the hypolipidemic drug clofibrate which is a typical inducing agent for the CYP 4A family [4, 6, 7]. NADPH-cytochrome c-reductase and cytosolic reductases show azoreductase activity, too [7]. To prove CYP 4A activity in lymphocytes, lymphocyte cultures were treated with clofibrate. To inhibit CYP 4A, the lymphocytes were treated with metyrapone.

Additionally the influence of the lymphocyte stimulating mitogen concanavalin A on enzyme activity was determined. Concanavalin A does not bind to antigene specific receptors but to carbohydrate structures which are located on all lymphocytes. It was shown that concanavalin A, despite the stimulation of proliferation, also increases CYP mediated demethylase activity in lymphocyte cultures [3].

2. Investigations, results and discussion

There are few papers reporting the use of lymphocytes for studying the biotransformation of drugs. As it is known that lymphocytes contain defined isozymes of the CYP-system they have been used more often as *in vitro*-model.

The yield of isolated lymphocytes was approximately 10^7 cells per spleen, and the viability tested by trypan blue exclusion was over 90%. The isolated lymphocytes were a mixture of B- and T-lymphocytes. Lymphocytes normally do not proliferate in culture, but by adding mitogens like the glycoprotein phytohaemagglutinin or concanavalin A, lymphocytes proliferate and double during 72 h [3]. Therefore, it was necessary to count the cells after the incubation time again.

The turnover rates of 7-ER in lymphocyte cultures (Fig. 1) were measured cumulatively over 72 h. In contrast to human lymphocytes [8], rat lymphocytes seem to express CYP 1A1 without any induction. Contrary to Stephen et al. [9], who reported no 7-ER-*O*-deethylase activity in spleen lymphocytes, we found low but detectable activity.

7-ER-*O*-deethylase activity is possibly only due to CYP 1A1 activity, because CYP 1A1 but not CYP 1A2 could be detected by Western blot analysis even after induction with 3-MC [10]. When concanavalin A was added to the culture medium, enhanced enzyme activity in the lymphocyte cultures was observed.

Phenobarbitone (PB) is an inducer for CYP 2B1 and 2 [4], and therefore it was not surprising that the activity of

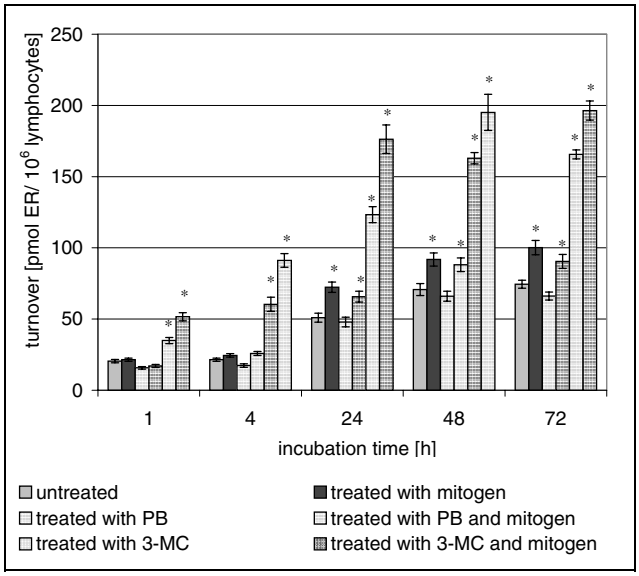

Fig. 1: Turnover of 7-ethoxyresorufin by spleen lymphocytes of the Wistar rat. Each value is the mean ± SD for ten trials. The marked values (*) are the turnovers of the treated lymphocyte cultures which were significantly different from untreated cultures for $p < 0.01$ using Student's t-test.

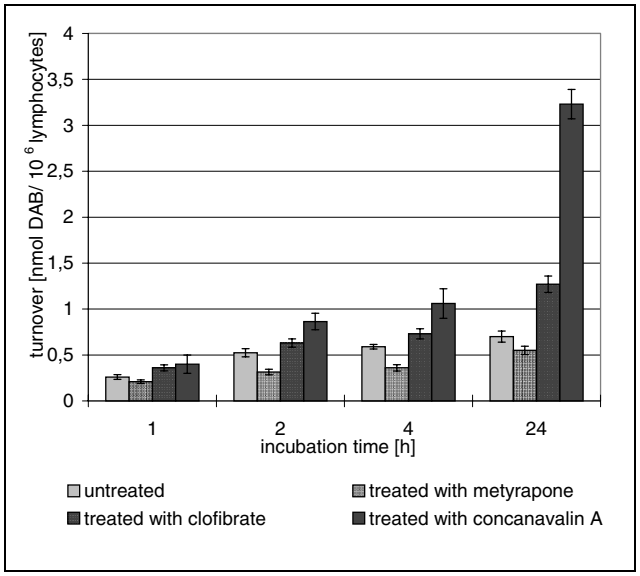

Fig. 2: Turnover of DAB by spleen lymphocytes of the Wistar rat. Each value is the mean ± SD for ten trials. All turnovers of the treated lymphocyte cultures were significantly different from untreated cultures for $p < 0.01$ using Student's t-test.

7-ER-*O*-deethylase was not enhanced but rather decreased. After incubation with concanavalin A the turnover could be increased again but did not reach the level of concanavalin A treated lymphocytes of non-treated rats. The highest induction was observed in lymphocytes of 3-MC-treated rats. Concanavalin A did not inhibit the induction of 3-MC as reported by Stephen et al. [9], using thymus lymphocytes and the inductor 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, but led to a further increase of CYP 1A1 activity as reported by Fung et al. [10], for blood lymphocytes of rats. Enzyme activity decreased in all cell cultures during 24 h, and there was only little further turnover after 48 h.

Reducing activities measured by the azoreduction of DAB were also found, whereby the highest activities were observed during the first two hours (Fig. 2). Again incubation with the mitogen concanavalin A enhanced enzyme activity and the turnover was increased more than 4-fold. Reductive activities concerning other substrates like *p*-nitrobenzoic acid could not be detected [3].

Azoreduction was increased 2-fold after treatment with the inducer clofibrate. This led to the conclusion that azoreduction has its origin in CYP 4A activity [11]. The supression of azoreductase activity by metyrapone, an inhibitor of CYP, corroborates this conclusion. Because only a partial supression of enzyme activity by metyrapone was observed, it has to be assumed that azoreductase activity is also due to cytosolic azoreductases.

With these model reactions the biochemical qualification of the *in vitro* test system 'lymphocyte culture' for studies of biotransformation was examined by estimating CYP 1A1 and CYP 4A activity and their inducibility. The data show that CYP 1A1 and CYP 4A is present in spleen lymphocytes, and activities were high enough for selective studies of oxygenation reactions. Both CYP enzymes were inducible by prototypic inducers and there was even a further induction after concanvalin A was added.

Apparently concanavalin A does not only stimulate the proliferation but also the activity of CYP 1A1, and even more the reductive activities. This leads to the conclusion that metabolic activity is dependent on the phase of the cell cycle.

3. Experimental

3.1. Chemicals

Lymphocyte culture media, supplements, Histopaque®-1077 and trypan blue were obtained from Sigma (St. Louis, MO, U.S.A.) and chemicals from Aldrich (Aldrich-Chemie, Steinheim Germany).

3.2. Animals and preparation of lymphocytes

Male Wistar rats (150–200 g) and standard rodent chow (Rezeptur R) were obtained from Tierzucht Schönewalde GmbH, Berlin. The rats were fed ad libitum.

For the isolation of lymphocytes rats were decapitated. The spleens were removed from the bodies and cooled in sterile 0.9% NaCl-solution, while the remaining fat was gently removed with a pair of scissors. Then the organ was cut into small pieces with a scalpel and filtered through a nylon mesh with a pistil. The homogenate was filled up to 10 ml with 0.9% NaCl-solution and then layered over 6 ml Histopaque®-1077 ($\rho = 1.077$ g/cm^3) and centrifuged for 30 min with $400 \times g$ to separate lymphocytes from other spleenocytes like macrophages [12]. The opaque interphase was suspended in 10 ml culture medium and again centrifuged for 10 min with $400 \times g$. The pellet was again suspended in culture medium and cell viability was tested with a 0.4% trypan-blue solution. To scrutinize, that only lymphocytes were maintained, a sample was stained with May-Grünwald solution and evaluated under the microscope. The lymphocyte suspension was topped up with the culture medium to a final concentration of 10^6 lymphocytes per ml and the cells were cultivated in 25 cm^2 flasks in a CO_2 incubator at a temperature of 37 °C as suspension culture. The medium consisted of RPMI 1640 supplemented with 10% fetal bovine serum and 25 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethane sulfonic acid. After all incubation times lymphocytes were counted with a Casy 1 Cell Counter Model TT.

3.3. Induction and inhibition

To induce CYP in lymphocytes Wistar rats were administered daily 50 mg/kg bodyweight i.p. PB or 3-MC solved in DMSO over 5 days before rats were decapitated [3]. Control animals received the corresponding volume of DMSO. Treatment with 60 μg/ml concanavalin A, 0.8 mM metyrapone or 0.8 μM clofibrate solved in DMSO was directly carried out by addition to the medium of the lymphocyte culture.

3.4. Ethoxyresorufin-O-deethylase

To start the assay 8 μM 7-ER were added to the medium. The presence of 10 μM dicumarol prevents further metabolism of resorufin formed by the cytosolic enzyme diaphorase [5]. At relevant times 2 ml samples were withdrawn and fluorescence was directly measured using a Shimadzu RF 5001 spectrofluorometer with 530 nm excitation and 583 nm emission filters. A standard curve of resorufin was prepared in the culture medium and standards were processed as described for samples. The limit of quantification of this fluorescence method was 22 pmol/ml. The repeatability was 2.2% and the accuracy 1.6%.

3.5. 4-(N,N-dimethylamino)azobenzene-reductase

DAB (0.2 mM) was added to cells and incubated for 1, 2, 4 and 24 h. Samples (0.5 ml) were added to 0.1 ml 1 N NaOH. The amine product, 4-(*N,N*-dimethylamino)aniline, was detected by the method described by Huang et al. [13]. These samples were extracted by vortexing twice with 1 ml of hexane for 30 s. Hexane layers were united and 1 ml was transferred to tubes containing 1 ml of 0.1 M sodium acetate buffer, pH 4.0. The amino product was extracted into this buffer by vortexing for 30 s. After centrifugation the hexane layer was carefully removed and 10 μl of fluorescamine solution (2 mg/ml in anh. acetone) were added. The fluorescence of the product was measured after 30 min with excitation and emission wavelengths of 410 and 500 nm. A standard curve of 4-(*N,N*-dimethylamino)aniline was prepared in culture medium processing as described above. The limit of quantification was 0.023 nmol/ml. The repeatability was 2.2% and the accuracy 3.2%.

The method used does not detect metabolites such as aniline and *p*-phenylenediamine or further metabolised products via reduction steps. Therefore the results are not an expression of total reduced metabolites [7, 14].

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