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The occurrence of carbonyl reduction in continuous cell lines emphasizes the essentiality of this metabolic pathway

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Using the ketone compound metyrapone (MPON) as a substrate for carbonyl reduction it has been verified for the first time that various permanent cell lines in culture express carbonyl reducing activity. This is even true for the dedifferentiated and fibroblastoid cell line V79, emphasizing the essentiality of this metabolic pathway. MPON reducing enzyme activities are located in the endoplasmic reticulum as well as in the cytoplasm of the cells. Compared to MPON-reductase in rat liver microsomes, no immunological homology to microsomal C2REV7 rat liver hepatoma cell MPON-reductase could be detected, indicating differences in antigenic determinants between the enzymes of the solid organ and respective cells in continuous culture.

Metyrapone (MPON); Carbonyl reductase; Reductive metabolism; Cell culture

1. INTRODUCTION

Metabolic reduction is the counterpart to oxidative pathways in the detoxication of many xenobiotics. This is especially true for lipophilic compounds bearing nitro or carbonyl groups [1]. Carbonylic substances often exert toxic effects on living matter, e.g. inhibition of growth or interferences with metabolic reactions of the cell [2] and, therefore, have to be metabolized immediately. Aromatic and aliphatic aldehydes and ketones frequently undergo carbonyl reduction as a first metabolic step prior to their further metabolism and elimination [3]. The enzymes mediating carbonyl reduction belong to the aldo-keto reductase family and comprise carbonyl reductase (EC 1.1.1.184), aldehyde reductase (EC 1.1.1.2) and aldose reductase (EC 1.1.1.21). Furthermore, enzymes like NAD(P)H:quinone-oxidoreductase (NQR, EC 1.6.99.2), dihydrodiol dehydrogenase (DDH, EC 1.3.1.20), and hydroxysteroid dehydrogenases might be involved in carbonyl metabolism. In addition to their contribution to the detoxication process carbonyl reductases seem to play significant roles in the biotransformation of physiologically occurring substances, such as steroids [4], prostaglandins [5] and biogenic amines [6]. Carbonyl reducing enzymes have been characterized in man and animal [7], but little is known about their expression in cells in continuous culture.

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In previous investigations we purified and characterized a carbonyl reductase from mouse liver microsomes using the ketone metyrapone (MPON) as substrate [8]. Moreover, we developed a method of HPLC analysis for direct determination of the reduced alcohol product metyrapol (MPOL) and, besides this, we raised antibodies against the microsomal mouse liver MPON-reductase to investigate immunological homologies to respective enzymes of other tissues and species.

Up to now MPON reducing enzymes have been found in mouse [8], rat [9], rabbit [10] and guinea pig [11] tissues. However, about their existence in permanent cell lines nothing is known. The present study has, therefore, been undertaken in order to establish whether continuous cell lines of human and rodent origin besides their expression of cytochrome P-450 isozymes [12], also express enzymes mediating carbonyl reduction, information of possible value also to other groups working on the enzymology of reductases.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Metyrapone was purchased from Fluka AG (Buchs, CH). Enzymatic tests were performed using $NADP^+$, G-6-P, G-6-P-DH from Boehringer Mannheim (Mannheim, FRG). For HPLC acetonitrile of HPLC-grade from E. Merck (Darmstadt, FRG) was used. Ham's F 12 and NCTC Cell Culture media were from Flow (Meckenheim, FRG), Dulbecco MEM-medium, penicillin, streptomycin and 4'6-diamidin-2-phenylindol-dihydrochlorid (DAPI) as well as diphenyl-hydantoin (DPH) from Boehringer Mannheim (FRG). All other chemicals were of highest commercially available grade.

2.2. *Cell culture*

2.2.1. Characteristics of cell lines used in this work

Dedifferentiated and fibroblastoid V79 cells were originally

isolated from Chinese hamster lung [12]. The cell line NCI-H322 was derived from a human lung tumor having morphological and functional features of Clara cells [13]. H4IIEC3/G$^+$ and C2REV7 are descendants of the Reuber H35 hepatoma cell line [14,15]. H4IIEC3/G$^-$ had been intermittently cultured in glucose-free medium [16] to select those cells being able to perform gluconeogenesis as a marker for liver-specific functions. C2REV7 originated in a selected clone, re-expressing liver-specific functions. The latter 3 cell lines are differentiated, i.e. they possess specific attributes of their tissue of origin [17,18].

10^7 cells of V79 and 4×10^7 cells of the other 3 lines were seeded in 100 mm Greiner plastic dishes or 25 cm^2 cell culture flasks. V79 and NCI-H322 were grown in DMEM medium completed with 10% (v/v) foetal calf serum; 100 u/ml penicillin and 100 μg/ml streptomycin at 37°C in an atmosphere of 5% CO_2/95% O_2. H4IIEC3/G$^-$ and C2REV7 were cultured in Ham's F12/NCTC 135 medium (1:1) with 5% (v/v) foetal calf serum (supplement of antibiotics and culturing was the same as before). Medium was changed daily beginning from the 3rd day after seeding. For experiments cells were grown to their optimum density, a parameter of experience.

All cell lines used in this study were free of mycoplasma as judged by staining with DAPI [19].

2.3. *Preparation of subcellular fractions*

The monolayers were rinsed twice with isotonic (1.15% KCl) Tris-HCl buffer, pH 7.4, scraped off with a rubber policeman in 1 ml buffer per plate and sedimented by centrifugation at 200 × g. After 10 s of homogenization with an Ultraturrax in the cold, the homogenate was centrifuged 10 min at 600 × g, 10 min at 12 000 × g and 60 min at 170 000 × g; all steps being carried out at 4°C. Remaining supernatant represents the cytosolic, the sediment the microsomal fraction, which was resuspended in Tris-HCl buffer pH 7.4.

2.4. *Incubations in cultured monolayers*

MPON, dissolved in medium, was added to a final concentration of 0.06, 0.4 or 1 mM to dishes having gained the optimum cell density. A 50 μl aliquot was taken from the medium after appropriate time intervals during the incubation period and the reaction was stopped by mixing it with 100 μl ice-cold acetonitrile. The samples were centrifuged in an Eppendorf centrifuge at 8000 × g for 6 min in the cold to sediment organic material and 20 μl of the supernatant served for HPLC-determination of MPON and MPOL.

Experiments to investigate a possible induction of carbonyl reducing activity by diphenylhydantoin (DPH) were carried out by adding 0.5 mM DPH for 48 h to the cells.

2.5. *Incubations with subcellular fractions*

The assay of MPON-reduction was performed by incubating 65 μl of biological material in a final volume of 75 μl at 37°C. After addition of 5 μl of a NADPH-regenerating system (final concentrations: $NADP^+$, 0.8 mM; G-6-P, 6 mM; G-6-P-DH, 0.35 U; $MgCl_2$, 3 mM) the reaction was started by adding 5 μl of 15 or 60 mM MPON (dissolved in water) to a final concentration of 1 or 4 mM. The reaction was stopped by transferring 15 μl of the incubation mixture into 45 μl ice-cold acetonitrile, proceeding further as described above. Respective controls were performed without biological material.

2.6. *Determination of metyrapone (MPON) and metyrapol (MPOL) by HPLC*

MPON and its reduced alcohol metabolite MPOL were detected on a reversed phase HPLC system, using an Octadecyl-Si 100 polyol (Serva, Heidelberg, FRG) matrix column (4.5 mm × 25 cm) and an eluent of 30% acetonitrile (v/v) in 30 mM phosphate buffer, pH 7.4. MPOL elutes at about 6.5 min and MPON at 10.0 min (flow rate: 1 ml/min) and are monitored at 254 nm with an UV monitor and HPLC integration software (BioRad, Munich, FRG).

2.7. *Determination of protein*

Protien determination was carried out according to Lowry et al. [20].

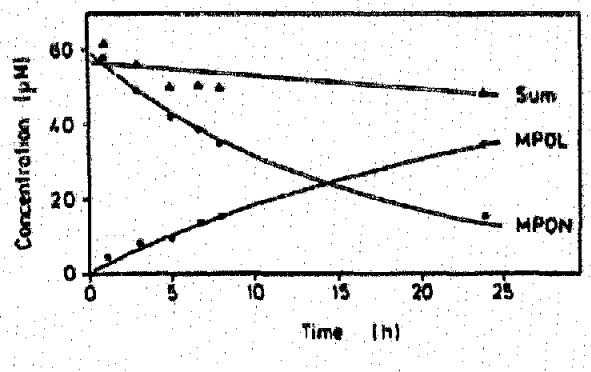

Fig. 1. The reduction of MPON in V79 monolayers in culture. The time course of changing concentrations of the reduced alcohol metabolite MPOL (○—○) and the substrate MPON (●—●) are shown. The correlation coefficient of the rectangular hyperbola for MPOL is $r^2 = 0.990$ and of the exponential decay for MPON $r^2 = 0.987$. MPON was added in culture medium to a final concentration of 60 μM. For comparison the time course of the concentration of the sum of MPON and MPOL is given (▲—▲); $n = 2$.

2.8. *SDS-polyacrylamide gel electrophoresis, immunoblotting and preparation of antibodies*

SDS-polyacrylamide gel electrophoresis, immunoblotting and preparation of antibodies against the MPON reductase from mouse liver microsomes were performed as described previously [21].

3. RESULTS

3.1. *Incubations with cells in culture*

During the same time course of all experiments with cells in culture the protein content of the cells changed only slightly (5–10%) per flask. The formation of the reduced metabolite metyrapol (MPOL) was thus mediated by a fairly constant amount of reducing enzymes. Control experiments without cells showed less than 5% of the activity with cells, which was at the limit

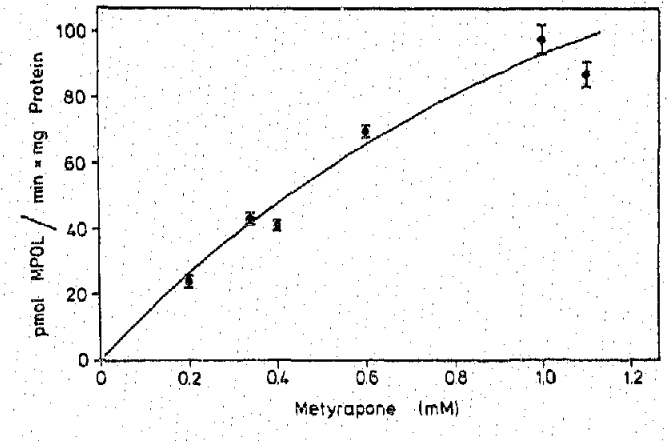

Fig. 2. Michaelis-Menten kinetic of MPON-reduction in NCI-H322 cells in culture. For details of incubation conditions see Section 2. Values are means ± SD, $n = 2$–12 ($n = 28$ altogether). Iteration analysis with KINPLT kinetic software gave a K_M of 1.73 ± 0.65 mM and a V_{max} of 252.5 ± 65.5 pmol/min·mg^{-1} protein. The correlation coefficient for linear regression was $r^2 = 0.93$.

of detection and which might be caused by components of the foetal calf serum in the culture medium. In accordance with [17] only 2–4 experiments were carried out for each time interval.

Fig. 1 shows the decreasing concentration of the substrate MPON and the increasing concentration of the substrate MPOL during a 24 h incubation. The non-linear time course of the corresponding curves indicates that at this substrate concentration reduction of MPON has not reached the maximum velocity. The sum of substrate and product concentrations decreases only very slightly during the incubation period, showing that other pathways of MPON-metabolism, such as N-oxidation [22], do not seem to be important.

In Fig. 2 the kinetics of MPON-reduction in the human lung tumor cell line NCI-H322 are demonstrated, which gave a calculated V_{max} of 253 pmol/mg min^{-1}.

Fig. 3 compares the specific activities of carbonyl reduction with varying substrate concentrations of MPON in 3 cell lines. The dedifferentiated, fibroblastoid cell line V79 revealed the highest activities. Using 0.06 and 0.4 mM MPON, in V79 it was twice as high as in NCI-H322 and C2REV7. These differences in enzyme activity disappear by increasing the MPON concentration up to 1 mM. Obviously, the maximum velocity of MPON-reduction can be further enhanced in NCI-H322 and C2REV7 with substrate concentrations higher than 1 mM, whereas in V79 the maximum seems to be reached. However, 4 mM MPON added to the cells had a lethal effect on V79 and NCI-H322 after 24 h of incubation.

3.2. *MPON-reduction in subcellular fractions*

Concerning the subcellular localization of carbonyl reductases, the cell line V79 and NCI-H322 showed higher specific activities in both cytosol and microsomes than the cell lines C2REV7 and H4IIEC3/G$^-$ (cf.

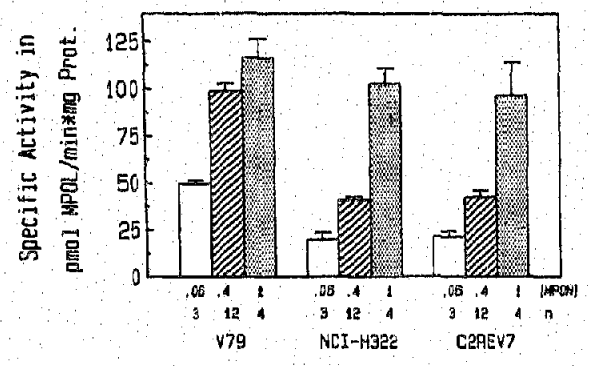

Fig. 3. The specific activity of MPOL formation of cells in culture. MPON was added in culture medium to final concentrations of 0.06, 0.4 and 1 mM. The incubation times were 24 h (left and right bar) and 48 h (centre bar). For details see Section 2. Values are means ± SD (number of experiments (n) and concentrations of MPON (mM) are given below the respective bars).

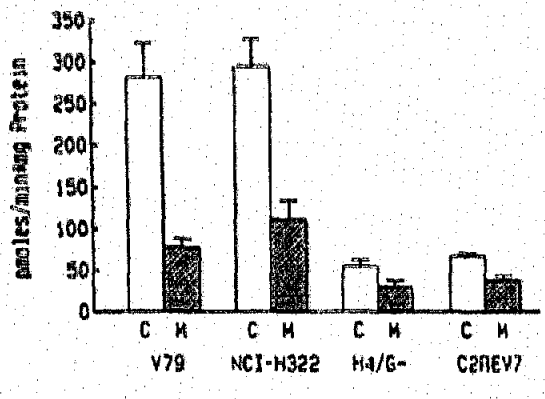

Fig. 4. The specific activity of MPOL formation in pmol/min · mg^{-1} protein in cytosol (C) and microsomes (M) of 4 cell lines. MPON was added to final concentration of 1 mM. The incubations were carried out with a NADPH-regenerating system in time intervals ranging from 15 to 90 min. For details see Section 2. Values are means ± SD, n = 16 of 4 individual preparations.

Fig. 4). In all 4 lines the cytosolic activities surpassed that of the respective microsomes.

3.3. *Immunoblot analysis*

Employing the immunoblot technique (Fig. 5) antibodies against the microsomal mouse liver MPON-reductase [23] crossreacted with its homologous antigen in rat liver microsomes, but not with any protein of the 4 cell lines, although equal amounts of protein (7.5 μg) were applied.

3.4. *Further investigations*

In all investigations there was no change in carbonyl reducing activity in relation to growth cycle or age of

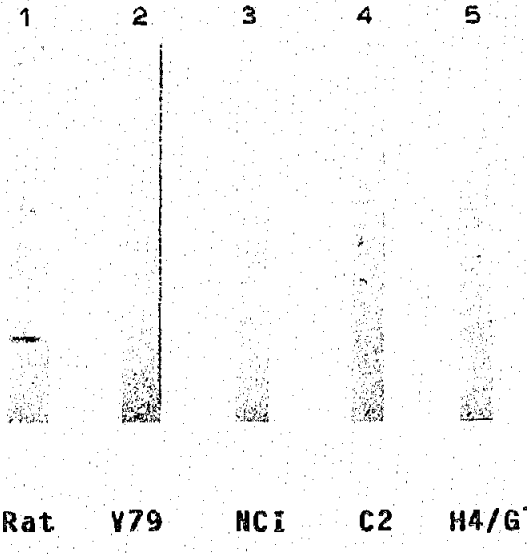

Fig. 5. Immunological comparison between MPON reducing enzymes of microsomes from rat liver (lane 1) and microsomes from cells of continuous culture (lane 2 = V79; lane 3 = NCI-H322; lane 4 = C2REV7; lane 5 = H4IIEC3/G$^-$). For details see Section 2.

the cells (number of passages). The inducibility of MPON-reduction was tested by adding the antiepileptic diphenylhydantoin (DPH), a well-known inducer of cytochrome P-450 and enhancer of MPON-decomposition [24] to cells in culture. In none of the 4 cell lines, neither in cytosol nor in microsomes could an enhancement of carbonyl reduction be detected.

4. DISCUSSION

The present study demonstrates that permanent cell lines express enzymes mediating the reductive biotransformation of carbonyl compounds. Using the ketone compound metyrapone (MPON) as substrate the cell lines V79, NCI-H322 and C2REV7 reach similar specific activities in culture incubations with MPON-concentrations of 1 mM.

Concerning the distribution of carbonyl reducing enzymes within the cell we concentrated on cytosolic and microsomal fractions as the main subcellular localization of biotransformation enzymes and found in all cell lines a cytosolic MPON-reducing activity surpassing that of the respective microsomes. Despite the occurrence of a microsomal form of MPON-reducing enzyme in the cultured cells no immunological crossreactivity was detected with antibodies against the MPON-reductase from mouse liver microsomes [23]. This could be due to a change in immunological homology, between rat liver as solid organ and C2REV7 rat liver hepatoma cells. In contrast, the MPON-reductase in rat liver microsomes was demonstrated to have immunological homologies with microsomal mouse liver enzyme (Fig. 5) [11].

In conclusion, carbonyl reduction seems to be an essential metabolic pathway, due to its maintenance in all continuous cell lines examined so far. This is even true for the dedifferentiated and fibroblastoid V79 cells, which have poor drug metabolizing enzyme activities [12] and in which high activities of MPON-reduction are found. Moreover, MPON-reducing enzymes are expressed independently of growth cycle and age (number of passages) of the cultured cells, thus again confirming the significance of carbonyl reduction as an essential metabolic pathway in normal cell physiology.

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