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CHARACTERIZATION OF CARBONYL REDUCING ACTIVITY IN CONTINUOUS CELL LINES OF HUMAN AND RODENT ORIGIN

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Abstract—Carbonyl reduction was investigated in the continuous cell lines V79, NCI-H322 and C2REV7 by using the ketone compound metyrapone as a substrate. Metyrapone reducing enzymes were characterized by evaluating the cosubstrate requirement and by testing the sensitivity of this reaction to specific inhibitors. All cell lines were found to produce metyrapol at a linear rate over a time course of at least 48 hr, when tested in cultured monolayers. In general, cytosolic metyrapone reduction exceeds microsomal activity several-fold in all three cell lines. Quercitrin turned out to be the strongest inhibitor in all fractions, except in NCI-H322 microsomes where it had no effect. Consequently, carbonyl reductase is suspected to be responsible for metyrapone reduction in the cytosol and microsomes of V79 and C2REV7 cells as well as in the cytosol of NCI-H322 cells. Simultaneous sensitivity towards quercitrin, dicoumarol, indomethacin and 5α-dihydrotestosterone in some cases points to the existence of different isozymes of carbonyl reductase. In NCI-H322 microsomes only dicoumarol and indomethacin decrease metyrapol formation, thus pointing to an isozyme of NAD(P)H:quinone-oxidoreductase. Concerning cosubstrate requirements metyrapone reducing enzymes show a strong preference for NADPH, thus confirming the involvement of carbonyl reductase in this reaction. In conclusion, carbonyl reduction of metyrapone in continuous cell lines is mediated by carbonyl reductases due to the common sensitivity towards the diagnostic inhibitor quercitrin and due to the strong preference for NADPH as cosubstrate. According to its maintenance in permanent cell lines carbonyl reductase seems to be an essential and constitutive enzyme, which probably fills an important role in normal cell physiology.

Cells in continuous culture are an attractive system to study the metabolism of xenobiotics under controlled conditions. They are of particular interest since they represent homogeneous populations and are generally readily available. One disadvantage is that they may change their metabolic activities [1–4] thus differing greatly in their enzyme pattern from cells derived from the solid organ. In the last few years there have been many reports about cell lines being capable of expressing a variety of the specific functions of the organ they originated from [4–7], which is true especially for cell lines from the liver, the organ containing the greatest accumulation of xenobiotic-metabolizing enzymes and expressing the highest activities. Efforts have been made to characterize in continuous cell lines the expression of phase I enzymes mediating oxidative drug metabolism [4, 5, 8] or phase II enzymes mediating drug conjugation [8, 9]. However, little is known about another important phase I reaction in biotransformation in permanent cell lines, which is the reduction of carbonyl compounds [10–12]. Carbonyl reduction is a metabolic pathway distributed widely in living matter [13, 14] and many endogenous compounds such as prostaglandins [15], biogenic amines [16] and steroids [17, 18], as well as xenobiotic aromatic and aliphatic aldehydes and ketones [13] are converted to the corresponding alcohols prior to their further metabolism and elimination. This is also true for pharmacologically relevant substances which are activated or inactivated by carbonyl reducing enzymes [19–22].

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). Enzymes like alcohol dehydrogenase (EC 1.1.1.1), NAD(P)H:quinone-oxidoreductase (EC 1.6.99.2), dihydrodiol dehydrogenase (EC 1.3.1.20) and hydroxysteroid dehydrogenases (3α-, 3β-, 17β-) may also be involved in the reductive metabolism of carbonyl compounds. Moreover, isozymes of dihydrodiol dehydrogenase and hydroxysteroid dehydrogenases were even thought to be identical to enzymes described previously as aldo-keto reductases [14, 23–29].

In previous investigations we demonstrated carbonyl reduction occurring in continuous cell lines, thus emphasizing the essentialness of this metabolic pathway [11]. The present study has been undertaken in order to characterize the enzymes mediating carbonyl reduction in continuous cell lines derived from the liver and lung of rodents and humans. We used the ketone metyrapone as a model substrate (Fig. 1) and employed a sensitive HPLC method for direct determination of the reduced alcohol product metyrapol. The characterization was performed by evaluating the cosubstrate requirement of metyrapone reduction and by testing the sensitivity of this reaction to specific inhibitors of enzymes which come into question concerning the carbonyl

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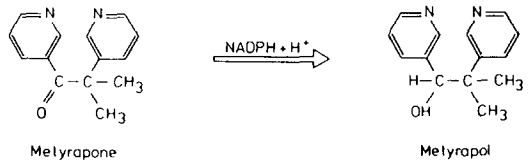

Fig. 1. Scheme of metyrapone reduction.

reduction of metyrapone. In particular, dicoumarol (inhibitor of NAD(P)H:quinone-oxidoreductase) [30], indomethacin (inhibitor of dihydrodiol dehydrogenase) [25], quercitrin (inhibitor of carbonyl reductase) [13], barbital and phenobarbital (inhibitors of aldehyde and aldose reductase) [13, 14], pyrazole (inhibitor of alcohol dehydrogenase) [31] as well as 5α-dihydrotestosterone (5α-DHT) (a substrate for 3α-hydroxysteroid dehydrogenase) were tested for their inhibitory potency towards metyrapol formation in the cytosol and microsomes of V79, NCI-H322 and C2REV7 cells, in order to determine the involvement of the respective enzymes in reductive metyrapone metabolism within these fractions. A preliminary account of this work has been presented [32].

MATERIALS AND METHODS

Chemicals. Metyrapone was purchased from Fluka AG (Buchs, Switzerland). Enzymatic tests were performed using NADH, NADPH, $NADP^+$, glucose-6-phosphate and glucose-6-phosphate dehydrogenase 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.), indomethacin from Sigma (Munich, F.R.G.), sodium barbital from E. Merck and 5α-DHT from Aldrich Chemie (Steinheim, F.R.G.). Ham's F12 and NCTC cell culture media were obtained from Flow (Meckenheim, F.R.G.) and Dulbecco's modified Eagle medium, penicillin, streptomycin and 4'6-diamidine-2-phenylindol-dihydrochloride (DAPI) from Boehringer Mannheim. All other chemicals were of the highest grade commercially available.

Cell culture. Characteristics of the cell lines used in this work are as follows: dedifferentiated and fibroblastoid V79 cells were originally isolated from Chinese hamster lung [3]. The cell line NCI-H322 was derived from a human lung tumor having the morphological and functional features of Clara cells [6]. C2REV7 cells are descendants of the Reuber H35 hepatoma cell line [1] and originated in a selected clone, re-expressing liver-specific functions [8]. The latter two cell lines are differentiated, i.e. possess specific attributes of their tissue of origin.

In 100 mm Greiner plastic dishes were seeded 10^5 cells of V79 and 4×10^5 cells of the other two lines. V79 and NCI-H322 cells were grown in Dulbecco's modified Eagle medium completed with 10% (v/v) foetal calf serum, 100 U/mL penicillin and 100 μg/mL streptomycin at 37° in an atmosphere of 5% CO_2/95% O_2. C2REV7 cells were cultured in Ham's F12/NCTC 135 medium (1:1) with 5% (v/v) foetal calf serum (supplement of antibiotics and culturing was as for the other two lines). Medium was changed daily beginning from the third 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 4'6-diamidine-2-phenylindol-dihydrochloride [33].

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/plate and sedimented by centrifugation at 200 *g*. After 10 sec of ultrasonification (Braun Melsungen Braunsonic 300 S) in the cold, the homogenate was centrifuged for 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°. Remaining supernatant represents the cytosolic, the sediment the microsomal fraction, which was resuspended in Tris–HCl buffer pH 7.4. Cytosols were dialysed prior to the enzyme assay against 1000 vol. of 50 mM sodium phosphate buffer, pH 7.4, to eliminate physiologically occurring pyridine nucleotides.

Incubations in cultured monolayers. Metyrapone, dissolved in medium, was added to a final concentration of 0.4 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 metyrapone and metyrapol.

Incubations with cell homogenate or subcellular fractions. The assay of metyrapone reduction was performed by preincubating 60 μL (65 μL in experiments concerning cofactor requirement) of biological material at 37°. Inhibitor studies were carried out by adding 5 μL of a stock solution of the respective inhibitor to give a final concentration of 1 mM. Inhibitors used were quercitrin, sodium barbital, phenobarbital, pyrazole, dicoumarol, indomethacin and 5α-DHT and were dissolved in 30 mM NaOH, except for 5α-DHT, which was dissolved in ethanol. Control velocities were determined in the presence of appropriate quantities of the solvents. Likewise, respective controls were performed without biological material. After the preincubation period of 1 min the reaction was started by adding 5 μL of 15 mM metyrapone (dissolved in water) to a final concentration of 1 mM and 5 μL of the respective cosubstrate (final concentrations: NADH 3.2 mM; NADPH 3.2 mM; NADPH-regenerating system: $NADP^+$ 0.8 mM, glucose-6-phosphate 6 mM, glucose-6-phosphate dehydrogenase 0.35 U, $MgCl_2$ 3 mM) to a final volume of 75 μL. The reaction was stopped by transferring 15 μL of the incubation mixture into 45 μL ice-cold acetonitrile. To assure linearity of the reaction during the incubation period several aliquots were

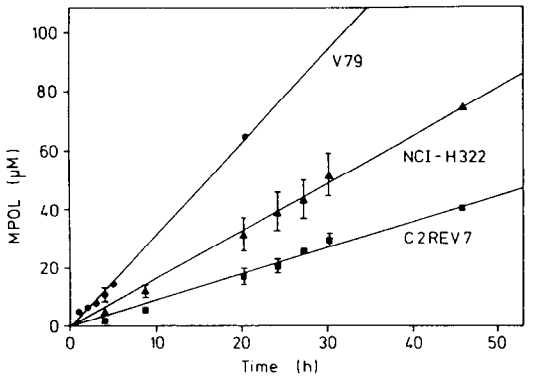

Fig. 2. The reduction of metyrapone in V79, NCI-H322 and C2REV7 monolayers in culture. Metyrapone, dissolved in medium, was added to a final concentration of 0.4 mM. A 50 µL aliquot was taken from the medium after appropriate time intervals during the incubation period and mixed with 100 µL ice-cold acetonitrile. After centrifugation 20 µL of the supernatant served for HPLC analysis of the reduced alcohol metabolite metyrapol (MPOL). Values are means ± SD. The correlation coefficients for linear regression (r^2) are 0.996 (V79), 0.995 (NCI-H322) and 0.985 (C2REV7).

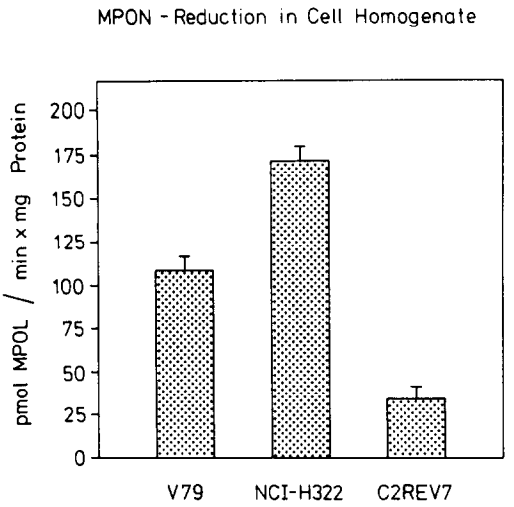

Fig. 3. The specific activity of metyrapol (MPOL) formation in homogenates of V79, NCI-H322 and C2REV7 cells. Metyrapone (MPON) was added to a final concentration of 1 mM. The incubations were carried out with a NADPH-regenerating system in time intervals between 15 and 90 min. For details see Materials and Methods. Values are means ± SD, N = 16–25 individual preparations.

taken in the time range between 15 and 90 min. The samples were centrifuged at 8000 *g* for 6 min in the cold to sediment organic material and 20 µL of the supernatant served for HPLC determination of metyrapone and metyrapol.

Determination of metyrapone and metyrapol by HPLC. Metyrapone and its reduced alcohol metabolite metyrapol were detected on a reversed phase HPLC system, using an Octadecyl-Si 100 polyol (Serva, Heidelberg, F.R.G.) matrix column (4.5 mm × 25 cm) and an eluent of 30% acetonitrile (v/v) in 30 mM phosphate buffer, pH 7.4. Metyrapol eluted at about 6.5 min and metyrapone at 10.0 min (flow rate: 1 mL/min), and both were monitored at 254 nm with a UV monitor and HRLC integration software (BioRad, Munich, F.R.G.).

Determination of protein. Protein determination was carried out according to Lowry *et al.* [34].

RESULTS

Metyrapone reduction in monolayers

V79, NCI-H322 and C2REV7 cells were tested for their ability to perform carbonyl reduction of metyrapone in cultured monolayers. Metyrapone, dissolved in medium, was added to a final concentration of 0.4 mM. Aliquots (50 µL) were taken from the medium after appropriate time intervals up to 48 hr and analysed by HPLC. Figure 2 shows that all cell lines were found to produce the reduced alcohol metabolite metyrapol at a linear rate over a time course of 48 hr. Interestingly, the dedifferentiated and fibroblastoid V79 cells expressed the highest activities, followed by NCI-H322 and C2REV7 cells. However, 4 mM metyrapone added to the monolayers had a lethal effect on all three cell lines after 24 hr of incubation.

Metyrapone reduction in cell homogenates

Velocity of substrate reduction in intact cells might not represent the true enzyme activities, because of possible influences of the plasma membrane on transmembraneous metyrapone–metyrapol fluxes as well as different levels of physiologically occurring cosubstrate pools within the cells. Therefore, metyrapone reduction was tested in homogenates of the cell lines in the presence of a NADPH-regenerating system. Figure 3 compares the specific activities of carbonyl reduction in homogenates of V79, NCI-H322 and C2REV7 cells. Whereas C2REV7 homogenate still has the lowest enzyme activity, NCI-H322 homogenate has a higher activity than that of V79 cells. Obviously, one of the reasons mentioned above is true for the NCI-H322 cell line.

Control experiments without biological material showed no carbonyl reducing activity, which confirms the dependence of metyrapol formation on enzymatic catalysis [35].

Incubations using 4 mM metyrapone as substrate revealed no differences in specific activities, thus confirming that 1 mM metyrapone is within the substrate saturation range.

Metyrapone reduction in subcellular fractions

In previous investigations it was shown that metyrapone reduction occurs in both cytosolic and microsomal fractions of liver cells [36–38] and that (at least) two distinct enzymes are responsible for metyrapone alcohol metabolite formation, being located in either of the two compartments [36, 38]. Considering these facts, homogenates of the three cell lines were separated into subcellular fractions and resulting microsomes and cytosol were tested for carbonyl reducing activities (Figs 4–6).

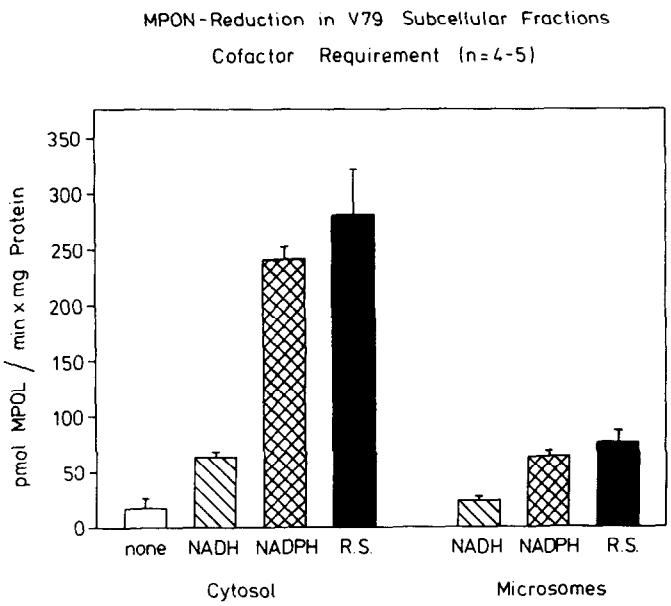

Fig. 4. Reduction of metyrapone (MPON) in V79 cytosol and microsomes with different cosubstrates. The specific activity was assayed in the presence of 1 mM metyrapone and either 3.2 mM NADH, 3.2 mM NADPH, a NADPH-regenerating system (R.S.) or without additional cosubstrate (none). Cytosols were dialysed prior to the enzyme assay against 1000 vol. of 50 mM sodium phosphate buffer, pH 7.4, to eliminate physiologically occurring pyridine nucleotides. Activity is expressed as pmoles metyrapol (MPOL) formed per minute and milligram of protein. Values are means ± SD of four individual preparations.

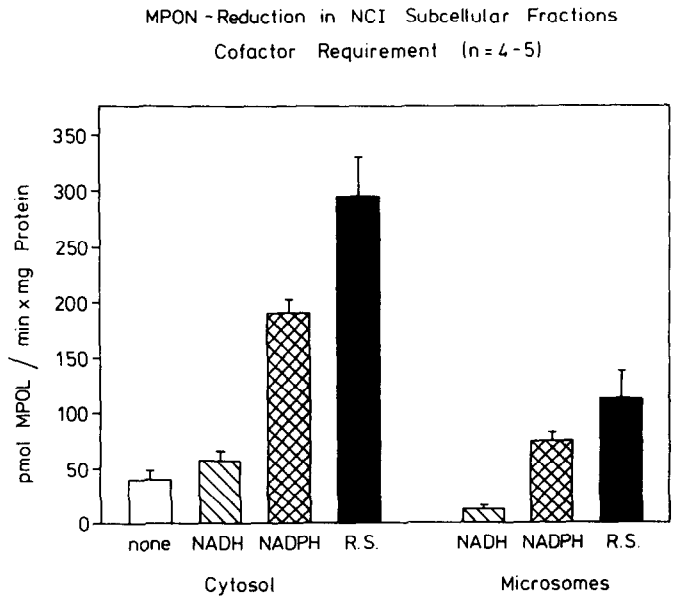

Fig. 5. Reduction of metyrapone (MPON) in NCI-H322 cytosol and microsomes with different cosubstrates. The specific activity was assayed in the presence of 1 mM metyrapone and either 3.2 mM NADH, 3.2 mM NADPH, a NADPH-regenerating system (R.S.) or without additional cosubstrate (none). Cytosols were dialysed prior to the enzyme assay against 1000 vol. of 50 mM sodium phosphate buffer, pH 7.4, to eliminate physiologically occurring pyridine nucleotides. Activity is expressed as pmoles metyrapol (MPOL) formed per minute and milligram of protein. Values are means ± SD of four individual preparations.

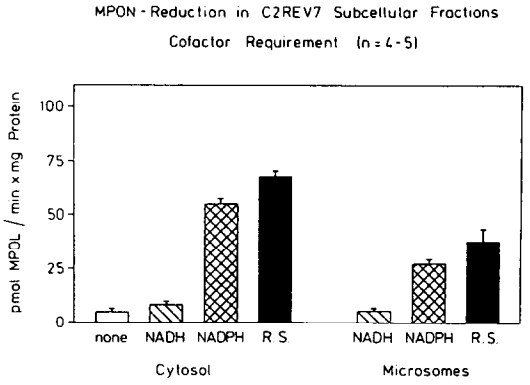

Fig. 6. Reduction of metyrapone (MPON) in C2REV7 cytosol and microsomes with different cosubstrates. The specific activity was assayed in the presence of 1 mM metyrapone and either 3.2 mM NADH, 3.2 mM NADPH, a NADPH-regenerating system (R.S.) or without additional cosubstrate (none). Cytosols were dialysed prior to the enzyme assay against 1000 vol. of 50 mM sodium phosphate buffer, pH 7.4, to eliminate physiologically occurring pyridine nucleotides. Activity is expressed as pmoles metyrapol (MPOL) formed per minute and milligram of protein. Values are means ± SD of four individual preparations.

In general, in all three cell lines cytosolic metyrapone reduction exceeded microsomal activity several-fold. In detail: using a NADPH-regenerating system as electron donor V79 cytosol shows 4-fold the specific activity compared to that in microsomes (282 vs 78 pmol/mg × protein), NCI-H322 cytosol shows 3-fold the specific activity compared to that in microsomes (296 vs 111 pmol/mg × protein) and in C2REV7 it still amounts to a nearly 2-fold preponderance (67 vs 38 pmol/mg × min).

Cofactor requirement of metyrapone reduction

Although aldo-keto reductases in principle are dependent on NADPH as cosubstrate, there are some reports on carbonyl reduction occurring with NADH [13, 17, 39]. Therefore, besides using a NADPH-regenerating system, carbonyl reduction was tested in the cytosol and microsomes of V79, NCI-H322 and C2REV7 cells in the presence of either NADPH or NADH in equal concentrations (Figs 4–6).

As expected, in all fractions metyrapone reduction shows its principal dependence on NADPH as cosubstrate. In C2REV7 cytosol and microsomes enzyme activities with NADH accounted for only 8.4% and 5.5% compared to that with NADPH (Fig. 6). Considering the relatively high enzyme activity without additional cosubstrate in NCI-H322 cytosol (possibly due to insufficient dialysis) it accounts for 19.3% in this fraction and 12.2% in NCI-H322 microsomes (Fig. 5).

In V79 cytosol and microsomes metyrapone reduction occurs to a considerable degree in the presence of NADH as cosubstrate, accounting for 25.7 and 47.3%, respectively, compared to that with NADPH (Fig. 4). Obviously, the dedifferentiated and fibroblastoid V79 cells are able to mediate carbonyl (metyrapone) reduction with NADPH as well as with NADH as cosubstrate. Nevertheless, highest specific activities were obtained using a NADPH-regenerating system as electron donor (Figs 4–6).

Inhibitor profile of metyrapone reduction

In addition to the classic aldo-keto reductases, alcohol dehydrogenase, dihydrodiol dehydrogenase, 3α-hydroxysteroid dehydrogenase and NAD(P)H:quinone-oxidoreductase were shown previously to be capable of carbonyl reduction [23–29]. To investigate the extent of their participation in carbonyl reduction of metyrapone in continuous cell lines the sensitivity of metyrapone reduction to specific inhibitors of the above-mentioned enzymes was tested in V79, NCI-H322 and C2REV7 cytosol and microsomes (Table 1).

Quercitrin, the specific inhibitor of carbonyl reductase, turned out to be the strongest inhibitor in all fractions, except that of NCI-H322 microsomes. It generally decreased the specific activities to about 50% compared to the uninhibited controls (in V79 cytosol even to 39%). Therefore, carbonyl reductase (EC 1.1.1.184) seems to be the enzyme mainly responsible for metyrapone reduction in these fractions. Indomethacin, the specific inhibitor of dihydrodiol dehydrogenase, turned out to be the strongest inhibitor in NCI-H322 microsomes (55% residual activity), suggesting that dihydrodiol dehydrogenases is the main metyrapone reducing enzyme there. Sensitivity towards indomethacin is also detectable in the cytosol of V79 and NCI-H322 cells (67% and 56% residual activity, respectively). Dicoumarol, the specific inhibitor of NAD(P)H:quinone-oxidoreductase, affects metyrapone reduction mainly in NCI-H322 cytosol (56% residual activity) and microsomes (64% residual activity), pointing to a possible participation of NAD(P)H:quinone-oxidoreductase in these fractions. Barbital and phenobarbital, the specific inhibitors of aldehyde reductase, had only small effects in V79 cytosol (75% and 83% residual activity). However, pyrazole, the specific inhibitor of alcohol dehydrogenase, had no effects in all fractions, thus excluding alcohol dehydrogenase from reductive carbonyl (metyrapone) metabolism. 5α-DHT, a substrate of 3α-hydroxysteroid dehydrogenase, decreased effectively enzyme activity in V79 cytosol (59% residual activity) and C2REV7 microsomes (60% residual activity) and scarcely in V79 microsomes (75% residual activity) and NCI-H322 cytosol (74% residual activity), thus indicating a possible involvement of 3α-hydroxysteroid dehydrogenase in reductive metyrapone metabolism. Surprisingly, addition of 1 mM dicoumarol as "inhibitor" increased the specific activity of metyrapone reduction in C2REV7 cytosol to 184% compared to the untreated control. This reproducible and time linear enhancement of enzyme activity is restricted to C2REV7 cytosol and has not been explained as yet.

DISCUSSION

Xenobiotics bearing a carbonyl function are

Table 1. Effects of inhibitors on carbonyl reduction of metyrapone in cytosol and microsomes of V79, NCI-H322 and C2REV7 cells

	Residual enzyme activity (%)*					
	V79		NCI-H322		C2REV7	
Inhibitor	Cytosol	Microsomes	Cytosol	Microsomes	Cytosol	Microsomes
Quercitrin	39.4	51.1	50.6	108.3	53.3	54.2
Indomethacin	66.5	83.2	55.6	55.4	88.2	86.7
Dicoumarol	84.3	85.6	56.3	63.5	184.3	96.2
5α-DHT	58.8	75.2	74.1	81.2	86.2	60.3
Barbital	74.6	87.3	97.8	87.9	86.7	89.3
Phenobarbital	82.6	n.d.	n.d.	n.d.	n.d.	n.d.
Pyrazol	94.5	96.9	94.8	93.6	97.7	93.9

* The enzyme activity was assayed in the presence of 1 mM metyrapone, a NADPH-regenerating system and 1 mM inhibitor. The percentages are calculated from uninhibited control experiments in the presence of appropriate quantities of the solvents. Values are means ± SD of 5–10 experiments.
5α-DHT = 5α-dihydrotestosterone.
n.d. = not determined.

chemically reactive and may have toxic consequences for living matter in that they interact with nucleophilic centers of physiological molecules thus interfering with metabolic reactions [40]. For maintaining regular cell function a rapid detoxification is therefore desirable. Investigations have established that most xenobiotic carbonylic substances are metabolized in mammalian species through reductive enzymatic pathways and presumably these enzymes function *in vivo* to eliminate chemically reactive and potentially toxic carbonyl compounds. Although oxidative xenobiotic metabolism is well described and has captured most investigative energies, reductive enzymatic pathways have not been compared adequately. This is especially true for enzymes mediating the carbonyl reduction of many aromatic and aliphatic aldehydes and ketones.

The present results demonstrate that permanent cell lines, such as V79, NCI-H322 and C2REV7, express enzymes mediating the reductive biotransformation of metyrapone, a model substrate for investigating carbonyl reducing enzymes [36–38, 41]. This is even true for the dedifferentiated and fibroblastoid V79 cells, which have poor drug-metabolizing enzyme activities and in which high activities of metyrapone reduction are found. Moreover, all three cell lines produce the reduced alcohol metabolite at a linear rate over a time course of 48 hr and respective enzymes are expressed independently of growth cycle and age (number of passages) of the cultured cells, facts which further confirm the constitutivity of these enzymes.

Deducing from the heterogeneous inhibitor profile, different isozymes seem to be involved in this reaction. Carbonyl reductase (EC 1.1.1.184) is the enzyme responsible for metyrapone reduction in the cytosol of all three cell lines as well as in the microsomes of V79 and C2REV7 cells, because of the sensitivity to the diagnostic inhibitor quercitrin. In addition to quercitrin, metyrapone reduction is also inhibited by indomethacin in V79 cytosol and by indomethacin as well as dicoumarol in NCI-H322 cytosol. Indomethacin, on the one hand, is known to be a specific inhibitor of dihydrodiol dehydrogenase (EC 1.3.1.20) [25] and dicoumarol of NAD(P)H:quinone-oxidoreductase (EC 1.6.99.2) [30]. On the other hand, several reports indicate that indomethacin and dicoumarol can also inhibit carbonyl reductase [14, 23, 26, 42–46]. Thus, simultaneous inhibition by quercitrin, indomethacin and dicoumarol corroborates the suggestion of carbonyl reductase mediating metyrapone reduction in the cytosol of V79 and NCI-H322 cells. Sensitivity of metyrapone reduction towards 5α-DHT additionally confirms carbonyl reductase mediating this reaction, since several reports indicate a relationship, and in some instances even identity, between hydroxysteroid dehydrogenases and carbonyl reductases [13, 23, 29, 47].

In NCI-H322 microsomes the sensitivity to indomethacin, together with the simultaneous insensitivity to quercitrin, points to a participation of dihydrodiol dehydrogenase in metyrapone reduction, which probably is not related to carbonyl reductase. Dicoumarol sensitivity in the same fraction indicates that NAD(P)H:quinone-oxidoreductase might also be involved.

Poor inhibitory sensitivity towards barbiturates indicates that both aldehyde reductase and aldose reductase have a minor role in metyrapone reduction in all fractions. Unlike carbonyl reductase, aldose reductase is also sensitive to barbiturates in addition to quercitrin [14]. On the other hand, aldehyde reductase is specifically inhibited by barbiturates [13]. Therefore, the absence of any significant inhibition by barbiturates indicates that neither aldose reductase nor aldehyde reductase are likely to be involved in metyrapone reduction.

Complete insensitivity towards pyrazole also excludes alcohol dehydrogenase from being involved in the carbonyl reduction of metyrapone in all three cell lines. Alcohol dehydrogenase occurs in multiple forms and in some instances is able to reduce aldehydes and ketones [48].

Concerning cosubstrate requirements metyrapone reducing enzymes show a strong preference for NADPH, thus confirming the involvement of carbonyl reductase in this reaction. In V79 cells NADH is also involved, although to a lesser degree. Nevertheless, highest specific activities were obtained using a NADPH-regenerating system as electron donor.

Surprisingly, addition of 1 mM dicoumarol as "inhibitor" increased the specific activity of metyrapone reduction in C2REV7 cytosol to 184% compared to the untreated control. This reproducible and time linear enhancement of enzyme activity is restricted to C2REV7 cytosol and has not been explained as yet.

To summarize, carbonyl reduction of metyrapone in continuous cell lines was demonstrated to be mediated by carbonyl reductases due to the common sensitivity towards the diagnostic inhibitor quercitrin and due to the strong preference for NADPH as cosubstrate. Additional sensitivity towards dicoumarol, indomethacin and 5α-DHT in some cases points to the existence of different isozymes of carbonyl reductase, which might be related to NAD(P)H:quinone-oxidoreductase, dihydrodiol dehydrogenase and hydroxysteroid dehydrogenase, respectively. In conclusion, due to its maintenance in permanent cell lines carbonyl reductase seems to be an essential and constitutive enzyme, which probably fills an important role in normal cell physiology.

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REFERENCES

1. Pitot HC, Peraino C, Morse A and Potter VA, Hepatomas in tissue culture compared with adapting liver *in vivo*. *Natl Cancer Inst Monogr* **13**: 229–242, 1964.
2. Bouhnik J, Cassio D, Coezy E, Corvol P and Weiss MC, Angiotensinogen production by rat hepatoma cells in culture and analysis of its regulation by techniques of somatic cell genetics. *J Cell Biol* **97**: 549–555, 1983.
3. Wiebel FJ, Roscher E and Hesse S, Metabolism of xenobiotics in cellular test systems: problems and prospects. In: *Alternatives to Animal Experiments in Risk Assessment* (Eds. Günzel P, Reinhardt C and Schiffmann D), pp. 85–92. Sala Druck, 1988.
4. Shean K and Paine AJ, Immunochemical quantification of cytochrome P450IA and IIB subfamilies in the livers of metyrapone-treated rats. *Biochem J* **267**: 715–719, 1990.
5. Wiebel FJ, Park SS, Kiefer F and Gelboin HV, Expression of cytochromes P-450 in rat hepatoma cells. *Eur J Biochem* **145**: 455–462, 1984.
6. Smith AC, McMahon JB, Schuller HM and Boyd MR, Phospholipid synthesis in small cell (SCCL) and non-small cell (NSCCL) carcinoma cell lines from human lung tumors. *Proc Am Assoc Cancer Res* **26**: 9–13, 1985.
7. Hirota K, Kawanishi T, Sunouchi M, Ohno Y, Takanaka A, Yamazoe Y, Kato R and Murakoshi Y, Induction of cytochrome P450c and P450d by metyrapone in the primary culture of rat hepatocytes. *Jpn J Biochem* **51**: 136–139, 1989.
8. Krupski G, Kiefer F and Wiebel FJ, Variability in the expression of xenobiotic-metabolizing enzymes during the growth cycle of rat hepatoma cells. *Xenobiotica* **15**: 781–787, 1985.
9. Wiebel FJ, Kiefer F, Krupski G and Schuller HM, Expression of glutathione-*S*-transferase and phenol-sulfotransferase, but not of UDP-glucuronosyl-transferase, in the human lung tumor cell lines NCI-H322 and NCI-H358. *Biochem Pharmacol* **35**: 1337–1343, 1986.
10. Le Bot MA, Begue JM, Kernaleguen D, Robert J, Ratanasavanh D, Airiau J, Riche C and Guillouzo A, Different cytotoxicity and metabolism of doxorubicin, daunorubicin, epirubicin, esorubicin and idarubicin in cultured human and rat hepatocytes. *Biochem Pharmacol* **37**: 3877–3887, 1988.
11. Gebel T, Maser E and Netter KJ, The occurrence of carbonyl reduction in continuous cell lines emphasizes the essentiality of this metabolic pathway. *FEBS Lett* **282**: 359–362, 1991.
12. Buller AL, Sharp RB and Penning TM, Characterization of dihydrodiol dehydrogenase in rat H-4IIe hepatoma cells. *Cancer Res* **49**: 6976–6980, 1989.
13. Felsted RL and Bachur NR, Mammalian carbonyl reductases. *Drug Metab Rev* **11**: 1–60, 1980.
14. Wermuth B, Aldo-keto reductases. In: *Enzymology of Carbonyl Metabolism 2* (Eds. Flynn TG and Weiner H), pp. 209–230. Alan R. Liss, New York, 1985.
15. Iwata N, Inazu N, Takeo S and Satoh T, Carbonyl reductases from rat testis and vas deferens. *Eur J Biochem* **193**: 75–81, 1990.
16. Smolen A and Anderson AD, Partial purification and characterization of a reduced nicotinamide adenine dinucleotide phosphate-linked aldehyde reductase from rat heart. *Biochem Pharmacol* **25**: 317–323, 1976.
17. Pietruszko R and Chen FF, Aldehyde reductase from rat liver is a 3α-hydroxysteroid dehydrogenase. *Biochem Pharmacol* **25**: 2721–2725, 1976.
18. Ikeda M, Hattori H and Ohmori S, Properties of NADPH-dependent carbonyl reductases in rat liver cytosol. *Biochem Pharmacol* **33**: 3957–3961, 1984.
19. Hermans JJR and Thijssen HHW, The *in vitro* ketone reduction of warfarin and analogues. Substrate stereoselectivity, product stereoselectivity and species differences. *Biochem Pharmacol* **38**: 3365–3370, 1989.
20. Hara H, Yamamoto H, Deyashiki Y, Nakayama T, Oritani H and Sawada H, Aldehyde dismutation catalyzed by pulmonary carbonyl reductase: kinetic studies of chloral hydrate metabolism to trichloroacetic acid and trichloroethanol. *Biochim Biophys Acta* **1075**: 61–67, 1991.
21. Inaba T and Kovacs J, Haloperidol reductase in human and guinea pig livers. *Drug Metab Dispos* **17**: 330–333, 1989.
22. Turner AJ and Hick PE, Metabolism of daunorubicin by a barbiturate-sensitive aldehyde reductase from rat liver. *Biochem J* **159**: 819–822, 1976.
23. Sawada H, Hara A, Nakayama T, Nakagawa M, Inoue Y, Hasebe K and Zhang Y, Mouse liver dihydrodiol dehydrogenases: identity of the predominant and a minor form with 17β-hydroxysteroid dehydrogenase and aldehyde reductase. *Biochem Pharmacol* **37**: 453–458, 1988.
24. Nakagawa M, Tsukada F, Nakayama T, Matsuura K, Hara A and Sawada H, Identification of two dihydrodiol dehydrogenases associated with 3(17)α-hydroxysteroid dehydrogenase activity in mouse kidney. *J Biochem* **106**: 633–638, 1989.
25. Penning TM, Mukharji I, Barrows S and Talalay P, Purification and properties of a 3α-hydroxysteroid dehydrogenase of rat liver cytosol and its inhibition by anti-inflammatory drugs. *Biochem J* **222**: 601–611, 1984.
26. Hara A, Taniguchi H, Nakayama T and Sawada H,

Purification and properties of multiple forms of dihydrodiol dehydrogenase from human liver. *J Biochem* **108**: 250–254, 1990.
27. Inazu N, Inaba N and Satoh T, Localization and regulation of ovarian carbonyl reductase in rats. In: *Enzymology and Molecular Biology of Carbonyl Metabolism 2* (Eds. Weiner H and Flynn TG), pp. 323–334. Alan R. Liss, New York, 1989.
28. Hara A, Usui S, Hayashibara M, Horiuchi T, Nakayama T and Sawada H, Microsomal carbonyl reductase in rat liver. Sex difference, hormonal regulation and characterization. In: *Enzymology and Molecular Biology of Carbonyl Metabolism* (Eds. Weiner H and Flynn TG), pp. 401–414. Alan R. Liss, New York, 1987.
29. Iwata N, Inazu N and Satoh T, The purification and characterization of NADPH-dependent carbonyl reductase from rat ovary. In: *Enzymology and Molecular Biology of Carbonyl Metabolism 2* (Eds. Weiner H and Flynn TG), pp. 307–321. Alan R. Liss, New York, 1989.
30. Lind C, Hochstein P and Ernster L, DT-diaphorase as a quinone reductase: A cellular control device against semiquinone and superoxide radical formation. *Arch Biochem Biophys* **216**: 178–185, 1982.
31. Li TK and Theorell H, Human liver alcohol dehydrogenase: inhibition by pyrazole and pyrazole analogs. *Acta Chem Scand* **23**: 892–902, 1969.
32. Gebel T, Maser E and Netter KJ, Reductive metabolism of the ketone metyrapone in continuous cell lines of human and rodent origin. *Naunyn Schmiedebergs Arch Pharmacol* **342** (Suppl): R10, 1990.
33. Boehringer Mannheim, 4′,6-Diamidine-2′-phenylindol-dihydrochloride (DAPI). In: *Cell Biology*. Boehringer Mannheim, F.R.G., 1990.
34. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
35. Maser E and Legrum W, Alteration of the inhibitory effect of metyrapone by reduction to metyrapol during the metabolism of methacetin *in vivo* in mice. *Naunyn Schmiedebergs Arch Pharmacol* **331**: 283–289, 1985.
36. Maser E, Characterization of microsomal and cytoplasmic metyrapone reducing enzymes from mouse liver. *Arch Toxicol* **13**: 271–274, 1989.
37. Maser E and Netter KJ, Purification and properties of a metyrapone reducing enzyme from mouse liver microsomes—this ketone is reduced by an aldehyde reductase. *Biochem Pharmacol* **38**: 3049–3054, 1989.
38. Maser E and Netter KJ, Reductive metabolism of metyrapone by a quercitrin-sensitive ketone reductase in mouse liver cytosol. *Biochem Pharmacol* **41**: 1595–1599, 1991.
39. Nakayama T, Hara A, Yashiro K and Sawada H, Reductases for carbonyl compounds in human liver. *Biochem Pharmacol* **34**: 107–117, 1985.
40. Agarwal DP and Goedde HW, Pharmacogenetics of aldehyde reductase (ALDH). *Pharmacol Ther* **45**: 345–371, 1990.
41. Maser E, Oppermann U, Bannenberg G and Netter KJ, Functional and immunological relationships between metyrapone reductase from mouse liver microsomes and 3α-hydroxysteroid dehydrogenase from Pseudomonas testosteroni. *FEBS Lett* **297**: 196–200, 1992.
42. Wermuth B, Purification and properties of an NADPH-dependent carbonyl reductase from human brain. *J Biol Chem* **256**: 1206–1213, 1981.
43. Sawada H, Hara A, Nakagawa M, Tsukuda F, Ohmura M and Matsuura K, Separation and properties of multiple forms of dihydrodiol dehydrogenase. *Int J Biochem* **21**: 367–375, 1989.
44. Iwata N, Inazu N and Satoh T, The purification and properties of NADPH-dependent carbonyl reductases from rat ovary. *J Biochem* **105**: 556–564, 1989.
45. Usui S, Hara A, Nakayama T and Sawada H, Purification and characterization of two forms of microsomal carbonyl reductase in guinea pig liver. *Biochem J* **223**: 697–705, 1984.
46. Katoh S and Sueoka T, Properties of carbonyl reductase activity of sepiapterin reductase, an enzyme involved in the biosynthesis of tetrahydrobiopterin. In: *Enzymology and Molecular Biology of Carbonyl Metabolism 2* (Eds. Weiner H and Flynn TG), pp. 381–395. Alan R. Liss, New York, 1989.
47. Hara A, Inoue Y, Nakagawa M, Naganeo F and Sawada H, Purification and characterization of $NADP^+$-dependent 3α-hydroxysteroid dehydrogenase from mouse liver cytosol. *J Biochem* **103**: 1027–1034, 1988.
48. Ziegler DM, Detoxication: oxidation and reduction. In: *The Liver: Biology and Pathobiology* (Eds. Arias IM, Jakoby WB, Popper H, Schachter D and Shafritz DA), pp. 363–374. Raven Press, New York, 1988.