Relationship between cytochrome P450 catalytic cycling and stability: fast degradation of ethanol-inducible cytochrome P450 2E1 (CYP2E1) in hepatoma cells is abolished by inactivation of its electron donor NADPH–cytochrome P450 reductase

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Ethanol-inducible cytochrome P450 2E1 (CYP2E1) involved in the metabolism of gluconeogenic precursors and some cytotoxins is distinguished from other cytochrome P450 enzymes by its rapid turnover (*in vivo* half-life of 4–7 h), with ligands to the haem iron, both substrates and inhibitors, stabilizing the protein. CYP2E1 is also known to have a high oxidase activity in the absence of substrate, resulting in the production of reactive oxygen radicals. We suggested that the rapid intracellular turnover of the enzyme may be partly due to covalent modifications by such radicals or to other changes during catalytic cycling, in which case the inhibition of electron supply from NADPH–cytochrome P450 reductase would be expected to stabilize the protein. Fao hepatoma cells, where CYP2E1 showed a half-life of 4 h upon serum withdrawal, were treated for 1 h with 0.3 μ M diphenylene iodonium (DPI), a suicide inhibitor of flavoenzymes,

which resulted in $\approx 90\%$ inhibition of the microsomal NADPH–cytochrome P450 reductase and CYP2E1-dependent chlorozoxzone hydroxylase activities. Subsequent cycloheximide chase revealed that the CYP2E1 half-life increased to 26 h. Neither the degradation rates of total protein, CYP2B1 and NADPH–cytochrome P450 reductase nor the cellular ATP level were affected by DPI under the conditions employed. These results demonstrate for the first time that the short half-life of CYP2E1 *in vivo* may be largely due to the rapid destabilization of the enzyme during catalytic cycling rather than to the intrinsic instability of the protein molecule.

Key words: diphenylene iodonium, endoplasmic reticulum, membrane proteins, protein degradation, reactive oxygen species.

INTRODUCTION

The cytochrome P450 superfamily comprises a number of haem-containing mono-oxygenases that, owing to different substrate specificities, catalyse the oxidative metabolism of a wide variety of lipophilic compounds. The cellular levels of individual cytochrome P450 isoenzymes are determined by their synthesis and degradation rates, both of which are subject to regulation. The cytochrome P450 forms differ considerably in their intracellular stabilities, the reported half-lives ranging from 5 to 37 h [2–6].

Ethanol-inducible cytochrome P450 2E1 (CYP2E1), with a half-life of 4–7 h [5–9], is the most unstable of all liver microsomal cytochrome P450 forms. However, its substrates (alcohols and ketones) and haem iron ligands (pyrazole, imidazole and related compounds) increase the half-life severalfold, protection from degradation being the major induction mechanism for CYP2E1 [5,8–10]. The molecular reasons for this short half-life are unknown, as also is the mechanism of substrate-offered protection. One possibility is that the CYP2E1 molecule possesses some unique structural features which expose it to the degradation machinery of the cell. It was shown previously in this laboratory that enhanced CYP2E1 degradation observed in primary hepatocytes on the addition of glucagon or cAMP correlates with increased phosphorylation on Ser¹²⁹, with substrates and ligands inhibiting both the degradation and phosphorylation [11,12]. This mechanism does not, however, explain the intrinsic instability of CYP2E1 in the absence of hormonal stimulation. It is also unclear whether phosphorylation is the

primary trigger of degradation. An alternative possibility is that degradation is initiated by some event during CYP2E1 catalytic cycling, so that the explanation for enzyme instability should be sought in the peculiarities of its catalytic functioning.

Recently we demonstrated that CYP2E1 undergoes rapid degradation in serum-deprived Fao rat hepatoma cells and, in line with previous findings, can be protected by the substrates and ligands [8]. In the present study we used this system to find out whether CYP2E1 catalytic cycling has a bearing on its fast degradation. To this end we blocked the supply of reducing equivalents to CYP2E1 by treating the cells with DPI, a suicide inhibitor of certain flavin oxidoreductases, including NADPH–cytochrome P450 reductase [13–17], and studied the effect of this treatment on the CYP2E1 degradation rate in the cell. We found that reductase inhibition, which suppressed the catalytic cycling of CYP2E1, had a major influence on the turnover rate of this cytochrome P450 isoenzyme in the hepatoma cells.

EXPERIMENTAL

Materials

L-[³⁵S]Methionine (sp. radioactivity > 1000 mCi/mmol), Hybond-C nitrocellulose membranes and enhanced-chemiluminescence (ECL[®]) kit were purchased from Amersham. Minimum essential medium, PBS, penicillin/streptomycin, and foetal-bovine serum were from Life Technologies. F12 Coon's modification medium, DPI, firefly luciferase mix, leupeptin, PMSF, cytochrome *c* and cycloheximide were from Sigma. Protein A–

Abbreviations used: CYP, cytochrome P450 (the nomenclature employed for cytochrome P450 is that recommended in [1]); CYP2E1, ethanol-inducible cytochrome P450 2E1; DPI, diphenylene iodonium; bis/acrylamide, methylenebisacrylamide/acrylamide.

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conjugated horseradish peroxidase and methylenebisacrylamide/acrylamide (bis/acrylamide) solution were from Bio-Rad.

Cell culture

Fao cells derived from Reuber H35 rat hepatoma were maintained in F12 Coon's modification medium supplemented with 5% (v/v) foetal-bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin. The cells were grown on 35-mm-diameter Petri dishes in a 5%-CO₂ humidified incubator at 37 °C. The medium was changed once in 2–3 days, depending on cell density and always the day before the experiment, which was run when the cells reached 80–90% confluence.

Isolation of microsomal fraction

The cell medium was discarded, the monolayers were washed with 10 mM Hepes/0.25 M sucrose, pH 7.5, and the cells were harvested and frozen. After thawing, the cell suspensions were sonicated and centrifuged at 13 000 *g* for 10 min. The pellet was discarded and supernatant centrifuged at 105 000 *g* for 60 min to pellet the microsomal fraction. Routinely, each experimental point was represented by three replicate 35-mm-diameter dishes. The cells harvested from these dishes were pooled, and the obtained microsomal pellet was resuspended in 0.2 ml of 10 mM Tris/HCl, pH 7.5.

Assay of microsomal NADPH–cytochrome P450 reductase

The activity of NADPH–cytochrome P450 reductase in the microsomal fraction was measured spectrophotometrically using cytochrome *c* as an electron acceptor [18] and expressed as percentage of that in the untreated control. Cytochrome *c* reduction was monitored at 550 nm and 30 °C. The incubation mixture contained 0.3 M potassium phosphate buffer, pH 7.6, 50 µM cytochrome *c*, 50 µM NADPH, and 25 µg/ml microsomal protein. One unit of activity was defined as the amount of microsomal protein catalysing the reduction of 1 nmol of cytochrome *c*/min under the above conditions.

Degradation assay of CYP2E1, CYP2B1 and NADPH–cytochrome P450 reductase

The cells were treated with 0.3 µM DPI for 1 h in the presence of serum and then supplied with fresh, serum-free, medium without DPI and with 10 µg/ml cycloheximide. Previously we showed that protein synthesis in Fao cells is entirely blocked under these conditions [8]. After incubation for indicated periods of time the cells were harvested and microsomes isolated as described above. The equal volumes of microsomal suspension obtained from different time points of the cycloheximide chase were subjected to SDS/PAGE in a Bio-Rad Mini-Protean II cell [7.5% (w/v) bis/acrylamide], with the volume calculated so that the zero-time control should contain 15 µg of microsomal protein. After electrophoresis the proteins were transferred on to a nitrocellulose membrane in the Towbin buffer system [19]. After completion of transfer, the membrane was dried, re-soaked in 50 mM Tris (pH 7.5)/0.2 M NaCl/0.05% (v/v) Tween 20 (NaCl/Tris/Tween), blocked in NaCl/Tris/Tween containing 5% (w/v) non-fat dry milk for 1 h, incubated for 1 h with anti-CYP2E1 [20], anti-CYP2B1 [21] or anti-reductase sera [22] and then for another 1 h with Protein A-conjugated horseradish peroxidase, all diluted 1:1000 in NaCl/Tris/Tween containing 1% (w/v) milk. The bands were visualized using an enhanced chemiluminescence kit (Amersham). Band densities were measured on a Personal Densitometer (Molecular Dynamics) and expressed as percentage of zero-time controls.

Assay of total protein degradation

Total protein degradation was monitored by using a pulse–chase technique. The cells were preincubated for 1 h in methionine-free Minimum Essential Medium supplemented with 5% (v/v) dialysed foetal-bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin. The cells were then labelled for 1 h with 25 µCi/ml [³⁵S]methionine in the same medium and chased in serum-free Coon's F12 medium containing 10 µg/ml cycloheximide. When the effect of DPI was to be studied the compound was present at a concentration of 0.3 µM during the methionine pulse. After indicated periods of time the cells were washed with PBS and extracted in the cold with 50 mM Tris/HCl (pH 8)/150 mM NaCl/1% (v/v) Triton X-100/1 mM PMSF/1 mM EDTA/10 µg/ml leupeptin. Radioactivity in the extracts, 98% of which was trichloroacetic acid-precipitable, was measured and expressed as percentage of the initial radioactivity.

ATP assay

To measure cellular ATP content the cell monolayers were extracted with 10% (w/v) trichloroacetic acid, and aliquots of the extracts were subjected to firefly-luciferase chemiluminescence assay (Sigma).

CYP2E1 substrate-binding spectra

Ethanol binding was monitored spectrophotometrically by monitoring the magnitude of high-to-low spin transition in CYP2E1. The difference spectra were recorded on a Perkin–Elmer Lambda 5 spectrophotometer at 22 °C in 50 mM potassium phosphate buffer, pH 7.4, containing 2.8% (w/v) glycerol and 1 µM purified CYP2E1 over the wavelength range 350–500 nm. The absorbance difference between the peak at 414 nm and the trough at 384 nm was used as a measure of the amount of substrate-bound enzyme. CYP2E1 was isolated from the liver of starved/acetone-treated rats as described previously [20].

Assay of chlorzoxazone hydroxylation

Chlorzoxazone 6-hydroxylase activity was measured in the microsomal fraction using an HPLC assay [23].

Assay of H₂O₂ formation in liver microsomes

Rat liver microsomes at a concentration of 0.3 mg/ml were incubated at 37 °C with 0.3 mM NADPH in 50 mM Tris/HCl buffer, pH 7.4, containing 150 mM KCl, 10 mM MgCl₂ and 1 mM NaN₃ in the absence and presence of 0.3 µM DPI. Aliquots were taken at specified time points and assayed for H₂O₂ content by the ferrithiocyanate method [24]. Microsomes were isolated from the livers of starved and acetone-treated rats as described previously [20].

RESULTS

Effect of DPI on microsomal NADPH–cytochrome P450 reductase and CYP2E1 activities

As seen from Figure 1(A), exposure of Fao cells to DPI caused the time- and dose-dependent inactivation of the microsomal NADPH–cytochrome *c* reductase. A 1 h treatment with 0.3 µM DPI was sufficient to inhibit the activity by more than 90%, which caused the activity of CYP2E1 to decrease by 85% as estimated by the 6-hydroxylation rate of its specific substrate chlorzoxazone (results not shown). The withdrawal of DPI led to a gradual recovery of the reductase activity (Figure 1B). That was apparently due to the *de novo* synthesis of the enzyme, since

Figure 1 Effect of DPI on NADPH-cytochrome P450 reductase activity in Fao cells

The activity was measured in microsomes isolated from the cells which were treated as follows: **(A)** the cells were treated with 0.1 μM DPI (●), 0.3 μM DPI (▲), or 0.6 μM DPI (◆) for indicated periods of time; **(B)** the cells were treated with 1 μM DPI for 2 h, at which point DPI was withdrawn and the cells were chased in DPI-free medium for indicated periods of time; **(C)** cells pre-treated with 0.3 μM DPI for 1 h (●) or control cells (○) were chased in DPI- and serum-free medium in the presence of 10 $\mu\text{g}/\text{ml}$ cycloheximide. The points represent means $\pm$ S.E.M. for three independent experiments. The average reductase activity in microsomes from untreated cells (100%) was 17 ± 1.9 units/mg.

Figure 2 Effect of DPI on degradation of total protein and individual microsomal proteins in Fao cells

Cells pre-treated with 0.3 μM DPI for 1 h (●) or control cells (○) were chased in DPI- and serum-free medium in the presence of 10 $\mu\text{g}/\text{ml}$ cycloheximide for indicated periods of time. The degradation of total protein was followed by [^{35}S]methionine pulse-chase and that of individual microsomal proteins by immunoblot (see the Experimental section for details). The points represent means $\pm$ S.E.M. for three independent experiments. Shown below is a representative CYP2E1 immunoblot.

the recovery could be prevented by the addition of the protein-synthesis inhibitor cycloheximide at the point of DPI withdrawal (Figure 1C).

Effect of DPI on degradation of CYP2E1, other microsomal proteins and total protein

Figure 2 shows the effect of DPI on the intracellular turnover of CYP2E1, CYP2B1, NADPH-cytochrome P450 reductase and total cellular protein. In control cells CYP2E1 had a half-life of about 4 h, whereas, when the cells had been treated for 1 h with 0.3 μM DPI followed by its withdrawal and the addition of

10 $\mu\text{g}/\text{ml}$ cycloheximide, the half-life increased to 26 h. In contrast, the rate of total protein degradation measured using [^{35}S]methionine pulse-chase was not affected by DPI. The estimated half-life under the conditions used was 15 h both in the control and DPI-treated cells. The degradation of NADPH-cytochrome P450 reductase and another cytochrome P450 form, CYP2B1, showed no response to DPI treatment either.

Analysis of DPI binding to CYP2E1

Although the effect of DPI on CYP2E1 degradation was monitored after DPI withdrawal, control experiments were performed to rule out the possibility that residual DPI tightly bound to the

Figure 3 Hanes plot for concentration-dependence of ethanol-induced spectral changes of CYP2E1

The ethanol (EtOH) binding spectra were recorded in the absence (●) and presence (○) of 3 μ M DPI as described in the Experimental section, and the absorbance difference between the peak at 414 nm and the trough at 384 nm was plotted against ethanol concentration.

enzyme at the active site was responsible for the observed protection by a mechanism common to CYP2E1 substrates and inhibitors [10]. Since the protective effect of various compounds on CYP2E1 had previously been shown to correlate with the magnitude of the high-to-low spin shift of the haem iron [10], we examined whether DPI can bring about such changes or compete with other ligands causing the spin shift. Figure 3 shows that DPI did not affect the magnitude of ethanol-induced high-to-low spin shift of the haem iron, even at a concentration of 3 μ M, which is an order of magnitude higher than the concentration used in the degradation experiments. No changes were detected in CYP2E1 difference spectrum on the addition of DPI alone to concentrations as high as 50 μ M (results not shown).

Figure 4 Effect of DPI on ATP content in Fao cells

(A) The cells were treated with 0.1 μ M DPI (▲), 0.3 μ M DPI (●) or 0.6 μ M DPI (■) for indicated periods of time. (B) Cells pre-treated with 0.3 μ M DPI for 1 h were chased in DPI- and serum-free medium in the presence of 10 μ g/ml cycloheximide for indicated periods of time. ATP contents were measured in trichloroacetic acid extracts by firefly-luciferase assay and expressed as percentage of zero-time controls. The average ATP content in control cells was 22.3 nmol/mg of protein. The points represent means $\pm$ S.E.M. for three independent experiments.

Effect of DPI on ATP content in the cells

Since NADPH-cytochrome P450 reductase is not the only flavoprotein known to be affected by DPI [13–17], it was important to make sure that any possible variation of cytochrome P450 degradation rate in this system was specifically due to the reductase inhibition. Of greatest concern in this respect is the ability of DPI to inhibit the mitochondrial NADH dehydrogenase [13], which may be expected to decrease the cellular ATP level and thus impair the functioning of ATP-dependent proteases whose possible involvement in cytochrome P450 degradation has been pointed out [9,25,26]. Figure 4(A) shows, however, that no appreciable changes in ATP level occurred at DPI concentrations below 0.6 μ M and treatment times shorter than 2 h. In cells treated with 0.3 μ M DPI for 1 h followed by a cycloheximide chase (Figure 4B), the ATP level varied only within 10 %. At the same time, this DPI dose and exposure time were sufficient to inhibit NADPH-cytochrome *c* reductase activity by $\approx$ 90 %, with no recovery observed during the subsequent 10 h cycloheximide chase (Figure 1C).

Effect of DPI on NADPH-cytochrome P450 reductase activity and H_2O_2 formation in liver microsomes

One of the reasons for the observed function–stability relationship for CYP2E1 can be the production of damaging reactive oxygen species by the active enzyme (see the Discussion). We therefore checked to see whether exposure to DPI indeed suppressed the formation of H_2O_2 in microsomes. Since CYP2E1 content in Fao cells is too low for CYP2E1-dependent H_2O_2 generation to be detected by conventional means, we used rat liver microsomes, where CYP2E1 was induced by treating the animals with acetone and depriving them of food. Figure 5 shows that incubation of the microsomes with NADPH in the presence of DPI leads to the rapid inactivation of NADPH-cytochrome P450 reductase and concomitant, although less pronounced, suppression of H_2O_2 formation.

DISCUSSION

In the present paper we address the question of whether the known instability of CYP2E1 in the cell is related to its catalytic cycling or is entirely due to structural factors. CYP2E1 inhibitors such as imidazole and methylpyrazole had previously been shown to be among substances protecting the enzyme against degradation [5,8,10]. That, however, allowed a twofold explanation, the possible mechanisms being either inhibition of catalysis or steric protection of some sensitive structural elements by these substances. Inactivation of NADPH-cytochrome P450 reductase blocks the supply of reducing equivalents to cytochrome P450, thus making it catalytically quiescent without changing the protein structure or introducing any ligands. This approach can provide more definite evidence on the dependence of CYP2E1 stability on its functioning. The results obtained demonstrate that the quiescent CYP2E1, in contrast with the catalytically active enzyme, does not undergo fast degradation in Fao cells. Neither total microsomal protein, nor individual proteins other than CYP2E1, responded to DPI treatment in the same way, demonstrating that the effect is not due to DPI inhibiting the cellular degradation machinery. This means that, as a structural entity, CYP2E1 is not a better substrate for the cellular proteases than such stable proteins as CYP2B1 and NADPH-cytochrome P450 reductase. Rather, an explanation for its observed instability in cultured cells and *in vivo* should be sought in the peculiarities of its catalytic functioning.

Figure 5 Effect of DPI on NADPH–cytochrome P450 reductase activity and H_2O_2 formation in liver microsomes

Rat liver microsomes at a concentration of 0.3 mg/ml were incubated at 37 °C with 0.3 mM NADPH in the 50 mM Tris/HCl buffer, pH 7.4, containing 150 mM KCl, 10 mM MgCl_2 and 1 mM NaN_3 in the absence (●) and presence (○) of 0.3 μM DPI. Aliquots were taken at specified time points and assayed for H_2O_2 content (---) and NADPH–cytochrome P450 reductase activity (—). Abbreviation: u, units.

One of such peculiarities is that CYP2E1 differs from other isoenzymes in its especially high oxidase activity in the substrate-free form [27,28]. This results in the production of large amounts of superoxide anions, H_2O_2 and hydroxyl radicals, CYP2E1 being the main source of these species in microsomes [30–32]. Ample evidence is available on the ability of reactive oxygen species, primarily hydroxyl radicals and superoxide anions, to cause the oxidative modification of proteins, with some of these modifications increasing the proteolytic susceptibility [33–35]. As the site of production of these species, CYP2E1 would also be the most exposed and likely target of their damaging action, with oxidative modification being the event triggering the degradation. The stabilizing effect of CYP2E1 inhibitors can therefore be accounted for by their slowing down enzyme turnover, which prevents the formation of such reactive species. Indeed, incubation of liver microsomes with NADPH and DPI slowed down the formation of H_2O_2 concomitantly with the inhibition of NADPH–cytochrome P450 reductase. The fact that H_2O_2 formation was inhibited to a lesser extent than the reductase might be due to the existence of reductase-independent (and, therefore, cytochrome P450-independent) pathways of H_2O_2 formation in the microsomes.

The difference in the effect of DPI on the two cytochrome P450 forms (CYP2E1 and CYP2B1) shows that destabilization during catalytic turnover is not what triggers the degradation of the latter. This agrees with the earlier results of Ronis and co-workers, who showed that the disappearance of immunoreactive CYP2E1 from rat liver microsomes was preceded by its inactivation, whereas that was not the case with CYP2B1 [36].

Interestingly, all substances reported so far to stabilize CYP2E1, both inhibitors and substrates, bring about type II or modified type II spectral changes, that is, high-to-low spin transition of the haem iron. With ethanol, propanol and DMSO a correlation has been demonstrated between their protective efficiency and the magnitude of the high-to-low spin shift [10]. A relationship has been shown to exist between cytochrome P450 redox potential and the spin state of the haem iron, with the high-spin form having a higher potential, which should favour electron transfer from the reductase [37–39]. Indeed, a positive

correlation exists between the magnitude of substrate-induced high-spin shift and NADPH oxidation rate in liver microsomes [40]. CYP2E1 is one of the only two known liver microsomal cytochrome P450 forms whose haem iron is high-spin in the substrate-free form [41], which, given the above spin–potential relationship, may be the reason for its high oxidase activity. The high-to-low spin transition may, therefore, be a common mechanism by which CYP2E1 substrates and ligands control the enzyme turnover and, therefore, stability. It should be borne in mind, however, that with mammalian cytochromes the spin control of the redox potential is not as strong as with bacterial forms [39], indicating that factors other than iron spin state can also determine the turnover rate.

One cannot also rule out the possibility that substrates and inhibitors bound in the active site, besides their effect on turnover, provide a steric protection against oxygen-derived radicals which are always formed in cytochrome P450-dependent reactions as a result of uncoupling. Indeed, ethanol inhibits CYP2E1 turnover in a reconstituted system and decreases the initial rate of its reduction by the reductase by only 20% [42,43]. Whether the magnitude of ethanol effect *in vivo* is the same is not known, but it might be that inhibition alone is not sufficient to account for its strong protective effect [8,10]. In this case protection from the reactive oxygen species, either purely steric or due to the known ability of alcohols and DMSO to act as free-radical traps, might be expected to contribute further to CYP2E1 stabilization. Alternatively, ligands can constrain the protein structure so as to prevent any destabilizing conformational changes which may occur during catalytic cycling in the absence of substrate.

Previously another two cytochrome P450 forms, CYP1A2 and CYP3A, have been shown to be stabilized in the liver cells by their substrates isosafrole [44,45] and triacetyloleandomycin [4] respectively. It is noteworthy that both of these stabilizers are converted by their specific cytochrome P450 forms into metabolites that form tight, catalytically inactive, complexes with the enzyme [46–48], suggesting that the function–stability relationship demonstrated in the present study for CYP2E1 may in fact be common for several cytochrome P450 forms.

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