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Cytochrome P450 CYP1A1 Accumulates in the Cytosol of Kidney and Brain and Is Activated by Heme

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ABSTRACT

Cytochrome P450 CYP1A1 is expressed in most tissues. In brain and kidney, its function remains unclear because its enzymatic activity is barely measurable. Here, we report on the localization of CYP1A1 in the cytosol of kidney and brain, as revealed by immunoblotting with anti-CYP1A1 antibodies and by 7-ethoxyresorufin deethylation (EROD). Hematin (8 μM) added in vitro to cytosol increased the EROD-activity 10-fold in brain olfactory bulb and 7-fold in kidney, presumably by reconstitution of apocytochrome. Succinylacetone, an inhibitor of heme biosynthesis, increased the ratio of cytosolic to microsomal EROD activity of transiently expressed CYP1A1 in COS-1 cells from 1:1 to nearly 6:1. This indicates a strong decrease of microsomal activity with increasing succinylacetone concentration. CYP1A1 activities correlated with CYP1A1 protein assessed by immunoblotting. We conclude that the availability of heme is a limiting factor of P450 function in extrahepatic tissue. Our data further suggest that reduced availability of heme limits the incorporation of P450s into brain endoplasmic reticulum. These observations are important when assessing the function of P450s in extrahepatic tissue.

Cytochrome P450 (P450) is the collective term for a large superfamily of heme-containing proteins that play an important role in the oxidative metabolism of numerous endogenous and foreign compounds (Nelson et al., 1996). Four of the P450-families, families 1 to 4, are involved in drug metabolism and are preferentially expressed in the liver. Drug-metabolizing P450 isoforms also occur in extrahepatic tissue and include CYP1A1 and CYP2A3 in breast tissue, CYP1A1, CYP2C23, and CYP4B1 in kidney (Lohr et al., 1998), and CYP2B1, CYP2C29, CYP2D4, CYP2E1, CYP4F, and CYP3A9 in the brain (Strobel et al., 1997). The P450 isoforms in brain are localized in defined neuronal or glial cell populations (Volk et al., 1995; Hedlund et al., 1996). Several of the P450s expressed in the brain are inducible by alcohol, neuroleptics, anticonvulsants, and endocrine factors (Volk et al., 1995; Hedlund et al., 1996; Rosenbrock et al., 1999; Meyer et al., 2001; Rosenbrock et al., 2001).

An important question regarding P450s expressed in brain is whether these enzymes are active in drug and xenobiotic metabolism (Strobel et al., 1997). Pharmaceuticals used in treatment of neurological and mental diseases (e.g., clozapine, tricyclic antidepressants, phenytoin) exert their function in specific brain regions and their local concentration may also depend on biotransformation via internal brain P450 pathways (Shahi et al., 1991; Volk et al., 1995; Riedl et al., 1998). However, even after induction of P450 isoforms in brain, investigations of brain tissue revealed no or only marginal metabolism of these drugs (Warner et al., 1998). These observations have led to the hypothesis that P450s in brain may have other functions than drug metabolism and be regulated differently than the liver enzymes.

Omiecinski et al. (1978, 1980) have described the activation of extrahepatic P450 monooxygenases by addition of hematin to tissue homogenates. P450 activities of brain, kidney, and testis homogenate showed an enhancement of up to 70-fold after addition of hematin when benzo[*a*]pyrene, 7,12-dimethylbenz[*a*]anthracene, 17β-estradiol, acetanilid, chrysene, and benzo[*e*]pyrene were used as substrates (Omiecinski et al., 1978, 1980). Addition of hematin did not enhance the corresponding enzymatic activities of liver homogenates. These data suggested that the availability of heme may regulate the function of P450 in extrahepatic tissue and that this regulation is different from liver.

In the present study, we have investigated the functional activation of CYP1A1 by hematin in mouse kidney and several regions of mouse brain. Cellular subfractions of these organs and tissues were examined, exposed to heme and specific enzymatic assays and immunological probes for

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ABBREVIATIONS: P450, cytochrome P450; ER, endoplasmic reticulum; PCR, polymerase chain reaction; SA, succinylacetone; DTT, dithiothreitol; EROD, 7-ethoxyresorufin-*O*-deethylase; NF, naphthoflavone; ECL, enhanced chemiluminescence; XTT, 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide-sodium salt.

CYP1A1 were applied. Moreover, CYP1A1 was transiently expressed in COS-1 cells, in which heme synthesis was inhibited. Our results suggest that reduced availability of heme limits the incorporation of CYP1A1 into endoplasmic reticulum (ER) of brain and possibly other extrahepatic tissue.

Materials and Methods

Animals. Male mice (age of 7 months) were used for the experiments. The animals were maintained in a 12-h light/dark cycle in a controlled environmental animal facility and had free access to standard rodent chow and tap water. The mice were anesthetized with sodium pentobarbital, the left ventricle of the heart was punctured, and the animal was perfused with cold isotonic saline to remove the blood from the organs. Livers, kidneys, testes, and brains were excised, subsequently frozen in liquid nitrogen and stored at −80°C until use. Some brains were sectioned into olfactory bulb, cortex, subcortical white matter, cerebellum, and brainstem.

Chemicals. 7-Ethoxyresorufin, resorufin, bovine serum albumin, NADPH, NADH, and the cytotoxicology assay kit were obtained from Sigma-Aldrich (Buchs, Switzerland). Glucose 6-phosphate, glucose-6-phosphate dehydrogenase, PCR free nucleotide premix, *Pyrococcus woesei*-kit (proofreading *Taq* DNA polymerase), and the DNA ladder were purchased from Roche Diagnostics (Mannheim, Germany). Hemin was supplied by Porphyrin Products (Logan, UT). The CYP1A1/2 inhibitory antibodies were from Gentest (Woburn, MA). Cell culture media, TRIzol-RNA preparation kit and Moloney murine leukemia virus reverse transcriptase were obtained from Invitrogen (Basel, Switzerland). Gel purification and DNA preparation kits were purchased from QIAGEN (Basel, Switzerland). For cloning, the pGEM-T easy vector (Promega-CatalysAG, Wallisellen, Switzerland) and the pcDNA1.1_Amp expression vector (Invitrogen BV, Groningen, The Netherlands) were used. *Taq* DNA polymerase and the terminator ready reaction mix were obtained from PerkinElmer Life Sciences (Rotkreuz, Switzerland). PCR primers were synthesized by Intron (Kaltbrunn, Switzerland). All other reagents were from commercial sources at the highest purity available.

Cell Culture. Monkey kidney COS-1 cells (American Type Culture Collection, Manassas, VA) were maintained in standard medium consisting of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 4.5 g/l D-glucose, 4 mM L-alanyl-L-glutamine, 1 mM Na-pyruvate, and 2500 mU/ml streptomycin/penicillin. Cells were grown in 100-mm culture dishes at 37°C in a 5% CO_2 humidified environment. Succinylacetone (SA; 10 μM-1 mM) was dissolved in culture medium and added to cells at 25% of confluence. Cells were treated with SA for 48 to 96 h. Potential toxicity of SA was tested with the Sigma XTT-assay as follows: 2.5×10^4 cells per well in a 96-well plate were treated with 0, 10, 100, and 500 μM SA. After 48 h of treatment, cell viability was measured spectrophotometrically at 450 nm by examining the formation of formazan resulting from XTT bioreduction. For transfection experiments, 2.5×10^6 cells (25% confluent) were used. Cells (107; 100% confluent) were used for analysis of EROD-activity and immunoblotting.

RNA Purification and cDNA Synthesis. Total RNA of frozen tissue was isolated using the TRIzol-RNA purification protocol (Invitrogen). RNA was reverse-transcribed in 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$, 200 μM concentration of each deoxynucleotide triphosphate, 200 U/μg RNA Moloney murine leukemia virus reverse transcriptase, 28 U of ribonuclease inhibitor (RNAsin; Promega) and 2 μM oligo[dT_{14}(A/C/G)] primer. The mixture was incubated at 40°C for 1h.

Generation of Mouse CYP1A1 Full-Length cDNA by PCR. Specific primers for the generation of mouse CYP1A1 full-length cDNA were designed (Kimura et al., 1984). The CYP1A1 forward primer (sense strand) corresponded to nucleotides 88 to 107 (5′-AGCCACCTAGATCATGCCT-3′), and the CYP1A1 reverse primer to nucleotides 1667 to 1687 (5′-TCCTGGACAGTCTAAGCCTG-3′). For nested PCR, the primer pair was for nucleotides 153 to 171 (5′-TGGCTGTCACCGTATTCT-3′) (forward primer) and nucleotides 1456–1474 (5′-TTGCCCAAACCAAAGAGA-3′) (reverse primer). Diluted cDNA (3 μl) was amplified by PCR (Biometra T-Gradient thermal cycler; Biolabo, Châtel-St-Denis, Switzerland). Separated PCR-products were extracted from the agarose gel and purified by the use of QIAGEN quick-spin columns according to the protocol of the manufacturer.

Expression of CYP1A1 cDNA in COS-1 Cells. PCR products were ligated into the pGEM_T Easy vector (1:1–3:1 molar ratio) and used to transform *Escherichia coli* strain XL-1 blue. Clones were grown on culture plates containing Leibowitz medium [1% (w/v) NaC1, 1% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 2% (w/v) bactoagar, pH 7.0] supplemented with 100 μg/ml ampicillin. Full-length CYP1A1 cDNA was subcloned in sense orientation into pcDNA1.1_Amp expression vector. CYP1A1 cDNA inserts were checked by cycle sequencing using the PCR Terminator Ready Reaction Mix (PerkinElmer Life Science) in a ABI-Prism 310 sequencing machine. CYP1A1-pcDNA1.1_Amp plasmid DNA was transiently transfected into COS-1 cells using the DEAE-Dextran transfection method in a 100-mm culture dish (Cullen, 1987). COS-1 cells were treated with SA in concentration of 0, 10, 100, and 500 μM 24 h before transfection. Cells were collected for experiments after 48 to 60 h. Transfection efficiency was evaluated by cotransfection of yeast β-galactosidase (Cullen, 1987).

Disruption of COS-1 Cells. COS-1 cells were scraped off plates at 100% confluence and washed with PBS at 37°C. After centrifugation at 2000*g* for 3 min at RT, cells were resuspended in 100 mM sodium phosphate buffer, pH 7.4, containing 20% glycerol, 0.2 mM EDTA, and 0.5 mM DTT. COS-1 cells were disrupted by sonication (three 5-s strokes, 12 μm) using a Branson Cell Disruptor (Dunbury, CC).

Homogenization of Mouse Tissue. Liver, kidney, and brain tissue were used for homogenization. Tissue was homogenized by a gentle manual method using a glass-on-glass (Dounce) homogenizer in a 7-fold excess of homogenization buffer (50 mM Na-phosphate, pH 7.4, 1 mM EDTA, and 0.5 mM DTT).

Subfractionation of Mouse Tissue and COS-1 Cell Homogenates. Homogenate was centrifugated at 9,000*g* (Omiecinski et al., 1980). The 9,000*g* supernatant was directly applied to ultracentrifugation at 170,000*g* (r_{max}) for 30 min at 4°C using a Beckman TLA120.2 rotor in a Beckman benchtop ultracentrifuge. The 170,000*g* pellet was designated as *microsomes,* the 170,000*g* supernatant as *cytosol*. In some cases an aliquot of the homogenate was subsequently applied to ultracentrifugation without prior 9000*g* centrifugation to minimize proteolysis. The resulting 170,000*g* pellet was designated as *membrane fraction* because under these conditions, microsomes are not separated from nuclear membranes and mitochondria. NADPH-cytochrome P450 oxidoreductase was used as marker to check membranous contamination of the cytosol (Näslund et al., 1988). Protein content of the subfractions was measured according to Lowry et al. (1951). All methods used were proved to generate high integrity subcellular organelles and cytosol with negligible membranous contaminations (data not shown).

Ethoxyresorufin-*O*-deethylase Assay. EROD was measured according to the method of Burke et al. (1985) using an endpoint fluorometric determination. Samples, reflecting a protein amount of 20 to 40 μg (liver), 100 to 500 μg (extrahepatic tissue), and 50 to 100 μg (COS-1 cells) were preincubated in 100 mM sodium phosphate buffer, pH 7.4, containing 20% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 5 mM $MgC1_2$, 1 mg/ml bovine serum albumin, 6 μM substrate, and 3.4 μg of purified rat cytochrome P450 oxidoreductase for 2 min at 37°C. NADPH (0.5 mM) was added and the reaction was allowed to proceed for 15 min or 2 h at 37°C in darkness for liver or extrahepatic tissue, respectively. The reaction was performed under 100% oxygen (Omiecinski et al., 1980). Freshly made hematin (Correia and Meyer, 1975) in concentrations from 4 to 100 μM was added to the reaction

mixture 5 min after starting the reaction. Incubation was stopped by addition of methanol and the mixture was centrifuged at 14,000*g* for 10 min. Fluorescence of the product and identically handled resorufin standards were measured with a Perkin-Elmer LS50B luminescence photometer (PerkinElmer Life Sciences, Switzerland). An identical set of samples boiled for 15 min at 95°C were used as blanks. The dependence of reaction velocity from either substrate-, heme- or from protein concentration was proved to be linear under the experimental conditions (data not shown). For inhibition studies, α-naphthoflavone (α-NF) was added in concentrations of 6, 20, and 100 μM to the assay. Inhibition with rat polyclonal CYP1A1/2 inhibitory antibodies (Gentest, Daiichi, Japan) was performed using 10 to 50 μl of antiserum added to the assay. Preimmune rabbit serum was used as control. In some cases, NADH was used as cofactor.

Immunoblot Using Monoclonal Antibody mab-1-7-1 against CYP1A1. Protein from subfractions of liver (5–20 μg per lane), extrahepatic tissue (250–500 μg), or COS-1 cells (50–100 μg) were precipitated with methanol and resolved on 9% SDS polyacrylamide gels. As a reference, microsomes from β-NF–treated female mice were used. Proteins were transferred to polyvinylidene difluoride membranes (Immobilon P; Millipore AG, Volketswil, Switzerland) in buffer (125 mM Tris, and 960 mM glycine). Incubations were performed with CYP1A1 monoclonal antibody mab-1-7-1 (dilutions: 1:500, 1:2,000) (Park et al., 1982), followed by exposure to horseradish peroxidase-conjugated IgG (goat anti-mouse) at a dilution of 1:10,000. The immunopositive bands were visualized with enhanced chemiluminescence (ECL; Amersham Biosciences, Dübendorf, Switzerland).

Heme Determination. Heme content was determined by measurement of protoporphyrin IX fluorescence (Carvalho et al., 1997). Frozen brain or liver tissue was crushed on dry ice and subsequently treated with 2 M oxalic acid (100 μl) at 95°C for 30 min. Samples were resuspended in 900 μl of cold (6°C) PBS, vortexed, and centrifuged at 15,000*g* for 15 min. Fluorescence emission in the supernatant was determined spectrofluorometrically (LS50B; PerkinElmer Life Sciences). Excitation and emission wavelengths were set to 405 and 600 nm, respectively. Background was evaluated by measuring fluorescence in nonboiled samples. A standard curve of protoporphyrin IX was run in parallel.

Results

Enhancement of EROD-Activity by Hematin in Total Brain, Kidney, and Liver. We used the 7-deethylation of ethoxyresorufin to test and localize the activation of CYP1A1 by hematin in vitro in cellular subfractions of total brain, kidney, and liver. Interestingly, our data revealed that EROD-activity of brain and kidney was highest in cytosol, whereas microsomes exerted no or only negligible activity (Table 1). Furthermore, addition of hematin (8 μM) increased cytosolic activity 2-fold in brain and 7.4-fold in kidney. Kidney microsomes seem not to play a major role in heme-regulated EROD-activity (Table 1). In the case of liver, EROD activity was almost completely restricted to microsomes. In accordance with previous work using other substrates (Omiecinski et al., 1980), microsomal activity could not be increased by hematin (Table 1). Only a small cytosolic EROD activity (~1% of microsomal activity) was detected in liver that was enhanced by hematin. Thus, the enhancing effect of hematin was restricted with great majority to the cytosol of kidney and brain.

Enhancement of EROD Activity in Brain Sections after Addition of Hematin. Olfactory bulb, cortex, and subcortical white matter (mainly hypothalamus and striatum) are the brain regions where CYP1A1 mRNA expression has been reported (Iscan et al., 1990; Schilter and Omiecinski, 1993; Doostzadeh et al., 1996; Huang et al., 2000). We therefore analyzed the hematin effect on EROD-activity in these selected brain regions. Expectedly, EROD-activity was traced to the cytosol in all cases (Table 1). Olfactory bulb showed highest brain EROD-activity. This was much more pronounced after enhancement of cytosolic EROD-activity by hematin (8 μM), as that of olfactory bulb was 5- to 10-fold higher than that of white matter and cortex (Table 1). As expected from total brain investigations, microsomes did not contribute to EROD.

TABLE 1

Formation of resorufin by 7-deethylation of 7-ethoxyresorufin in subfractions of mouse liver, kidney, total brain, and the following brain sections: olfactory bulb, cortex, and subcortical white matter. Incubations were performed with 6 μM 7-ethoxyresorufin and 8 μM hematin where indicated as described under *Materials and Methods*. Values are presented as means ± SEM and represent a pool of 3 animals in case of liver (five experiments), 4 animals in case of brain (total) and kidney (three experiments), and 13 animals in case of brain olfactory bulb, cortex and subcortical white matter (three experiments).

Organ or Brain Section	Subfraction		
	9000 S	Microsomes	Cytosol
	pmol resorufin formed/min/mg of protein		
Liver			
No hematin	5.46 ± 0.50	11.88 ± 3.05	0.13 ± 0.01
8 μM hematin	4.98 ± 1.60	7.02 ± 1.70	0.87 ± 0.21
Kidney			
No hematin	0.08 ± 0.001	0.05 ± 0.001	0.07 ± 0.003
8 μM hematin	0.23 ± 0.002	0.08 ± 0.004	0.50 ± 0.12
Brain (Total)			
No hematin	0.02 ± 0.002	N.D.	0.05 ± 0.008
8 μM hematin	0.07 ± 0.006	N.D.	0.08 ± 0.01
Olfactory bulb			
No hematin	0.02 ± 0.001	N.D.	0.02 ± 0.001
8 μM hematin	0.06 ± 0.001	N.D.	0.16 ± 0.005
Cortex			
No hematin	N.D.	N.D.	N.D.
8 μM hematin	0.01 ± 0.000	0.01 ± 0.000	0.02 ± 0.000
Subcortical white matter			
No hematin	0.01 ± 0.000	N.D.	0.01 ± 0.000
8 μM hematin	0.01 ± 0.000	0.01 ± 0.000	0.03 ± 0.000

N.D., not detectable (activity < 0.004 pmol resorufin formed/min/mg of protein).

Specificity of CYP1A1 for Deethylation of 7-Ethoxyresorufin in the Cytosol. A number of experiments were done to evaluate whether the enzyme catalyzing 7-deethylation of ethoxyresorufin in cytosol of mouse extrahepatic tissue is identical with CYP1A1. First, the influence of cofactors and inhibitors of monooxygenase reactions was investigated using mouse total brain cytosol to assess the hemoprotein character of this EROD. Presence of carbon monoxide completely inhibited the reaction and product formation did not occur in the absence of cofactor NADPH or NADH. Second, the potency of the CYP1A1-specific inhibitor α-NF (Correira, 1995) to inhibit extrahepatic cytosolic EROD-activity was investigated using brain olfactory bulb (Fig. 1A). Resorufin formation decreased with increasing concentrations of inhibitor added. Using 100 μM α-NF (17fold excess of inhibitor), EROD activity of olfactory bulb cytosol was inhibited by 85%, assigning specificity of the reaction to CYP1A1. Third, cytosols from total brain, olfactory bulb, kidney, and liver were incubated with polyclonal inhibitory antibodies raised against purified rat CYP1A1 (Fig. 1B). Inhibition potency in mouse was checked previously using β-NF–induced liver microsomes (data not shown). Incubation with this antiserum revealed an effective inhibition of EROD in all samples investigated. In kidney and brain, already 20 μl of antiserum were able to inhibit EROD to 70 or ~50% of controls, respectively (Fig. 1B). Liver cytosol followed the same pattern as did brain and kidney. As expected, in olfactory bulb cytosol, EROD showed strongest inhibition by the antibodies. Already, 20 μl of antiserum decreased EROD to 15% of control activity. When using 50 μl of antiserum, EROD activity was undetectable (Fig. 1B).

A

Inhibition of EROD by α-Naphthoflavone			
Concentration of α-NF	*Ratio 7-ERF : α-NF*	*Cytosol Brain olfactory bulb*	*Microsomes Liver*
0 μM	*1:0*	0.4199±0.02	8.3207±1.603
6 μM	*1:1*	0.3675±0.02	not measured
20 μM	*1:3*	0.1989±0.018	0.4675±0.04
100 μM	*1:17*	0.0638±0.005	not measured

B

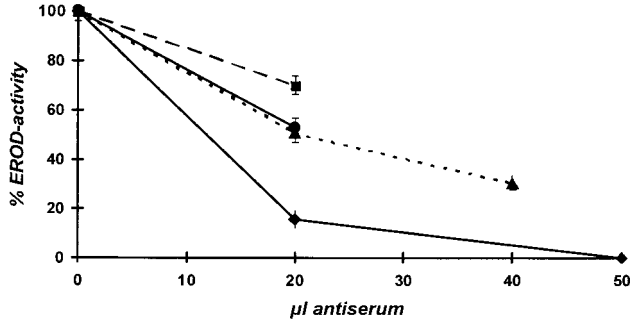

Fig. 1. Inhibition of EROD-activity by α-naphthoflavone (A) and by polyclonal antibodies against rat CYP1A1/2 (B). A, EROD-assay was performed as described under *Materials and Methods* with the indicated concentrations of α-NF. 7-Ethoxyresorufin (7-ERF) was used at a concentration of 6 μM. Assays were performed in the presence of 8 μM hematin. Values are expressed as picomoles of resorufin formed per minute per milligram of protein and represent mean ± S.E.M. of three independent experiments ($n = 3$) using a pool of 13 animals (brain olfactory bulb). B, cytosol of total brain (●), brain olfactory bulb (♦), kidney (■), and liver (▲) corresponding to 200 μg of protein were incubated with the indicated amounts of antiserum as described under *Materials and Methods*. Incubations were performed for 2 h at 37°C in the darkness with 8 μM hematin added to all samples. Preimmune rabbit serum was used as a control. Data points represent mean ± S.E.M. of 3 independent experiments ($n = 3$) from a pool of 2 animals (liver, kidney), 3 animals (total brain), and 13 animals (brain olfactory bulb).

Localization of CYP1A1 in Subfractions of Extrahepatic Tissue and Liver Determined by Immunoblot. Our experiments based on EROD support the presence of CYP1A1 in cytosol of extrahepatic tissue. To gather evidence for this concept, we performed immunoblots using a CYP1A1-specific monoclonal antibody (Park et al., 1982). The immunoreactive protein visible at a size corresponding to 59 to 60 kDa supports the presence of CYP1A1 in cytosol, because a protein of identical size is present in β-NF–induced liver microsomes (Fig. 2).

In (untreated) liver, CYP1A1 was exclusively localized in microsomes (Fig. 2). In extrahepatic tissue, the highest levels of CYP1A1 were detected in cytosol, as tested in cellular subfractions of kidney, total brain, and olfactory bulb (Fig. 2). No or only a weak immunosignal was found in the membrane fraction of these organs. Comparing these results with those obtained by performing EROD-assays (Table 1), the colocalization of cytosolic CYP1A1 and EROD is apparent.

Measurement of Heme in Mouse Tissue. Heme content and turnover in liver is under control of a regulatory heme pool, which adjusts heme saturation of P450s (Meyer et al., 1998). To evaluate the availability of heme in extrahepatic tissue, we analyzed heme content in liver, kidney, and brain

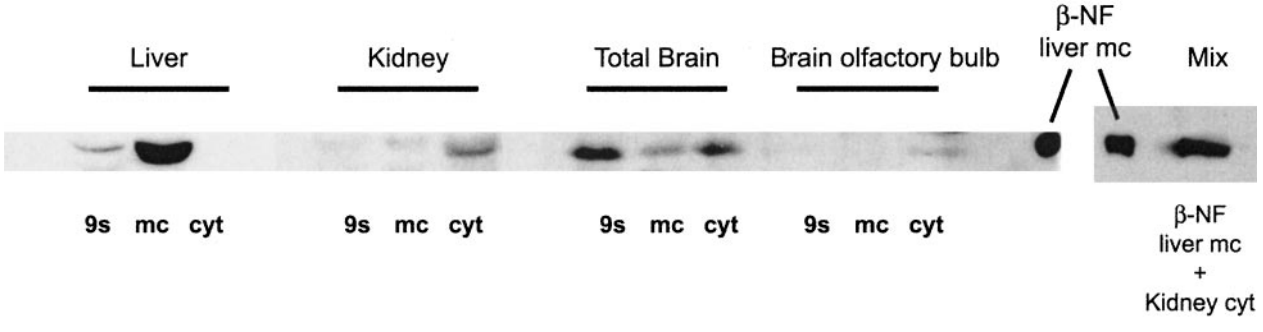

Fig. 2. Immunoblot with monoclonal antibody 1-7-1 raised against CYP1A1 (Park et al., 1982) in subfractions of liver, kidney, total brain, and olfactory bulb from male mice. Protein from 9000*g* supernatant (9s), microsomes (mc) and cytosol (cyt) were separated by SDS-PAGE as described under *Materials and Methods*. β-NF–treated liver microsomes and kidney cytosol were mixed to demonstrate the identical location of the recognized band. Immunosignals were visualized using ECL.

tissue (Table 2). In all extrahepatic tissues investigated, heme content was clearly diminished compared with liver. In kidney, 37.7% compared with liver heme was found. In brain, heme content was in similar range to that of kidney, with highest value in olfactory bulb, representing 39.6% of liver, lowest in central cortex and subcortical white matter (32.0–33.1% of liver) (Table 2).

TABLE 2

Heme content of mouse liver and brain sections

Frozen tissue was crushed and boiled in 2 M oxalic acid. Fluorescence emission of the resulting protoporphyrin IX was measured and quantitated against a protoporphyrin IX standard curve as described under *Materials and Methods*. Values represent means ± S.E.M.; $n = 3$.

Tissue	Heme Content	
	pmol/mg tissue	*% of liver value*
Liver	7.09 ± 0.78[a]	100.0 ± 11.0
Kidney	2.68 ± 0.38	37.7 ± 5.3
Olfactory bulb	2.81 ± 0.13	39.6 ± 1.8
Cortex	2.35 ± 0.58	33.1 ± 8.2
Subcortical white matter	2.27 ± 0.03	32.0 ± 0.4

Heterologous Expression of CYP1A1 in COS-1 Cells. We next tested the hypothesis that lack of heme maintains the CYP1A1 apoprotein in cytosol. Kidney derived COS-1 cells were transiently transfected with CYP1A1 cDNA in the presence of 10 μM-1 mM SA, an inhibitor of heme biosynthesis (Giger and Meyer, 1983). An expression plasmid was generated which contains a 1.6-kilobase full-length cDNA of CYP1A1 (Kimura et al., 1984), previously synthesized by RT-PCR and proved to have homology with CYP1A1 cDNA by cycle sequencing (data not shown).

After 48 to 60 h of transient transfection with the CYP1A1 expression plasmid, cells were subfractionated to generate a system corresponding to that of our investigations in mouse tissue. EROD-activity and protein expression were analyzed. As expected, a striking difference was found between SA-treated and untreated cells (Figs. 3 and 4). Compared with cytosolic activity, microsomal EROD decreased concomitant to increasing SA-concentration from a nearly 1:1 ratio in untreated cells to a 1:6 ratio in cells treated with 500 μM SA (Fig. 3A). Additionally, SA caused a significant reduction in

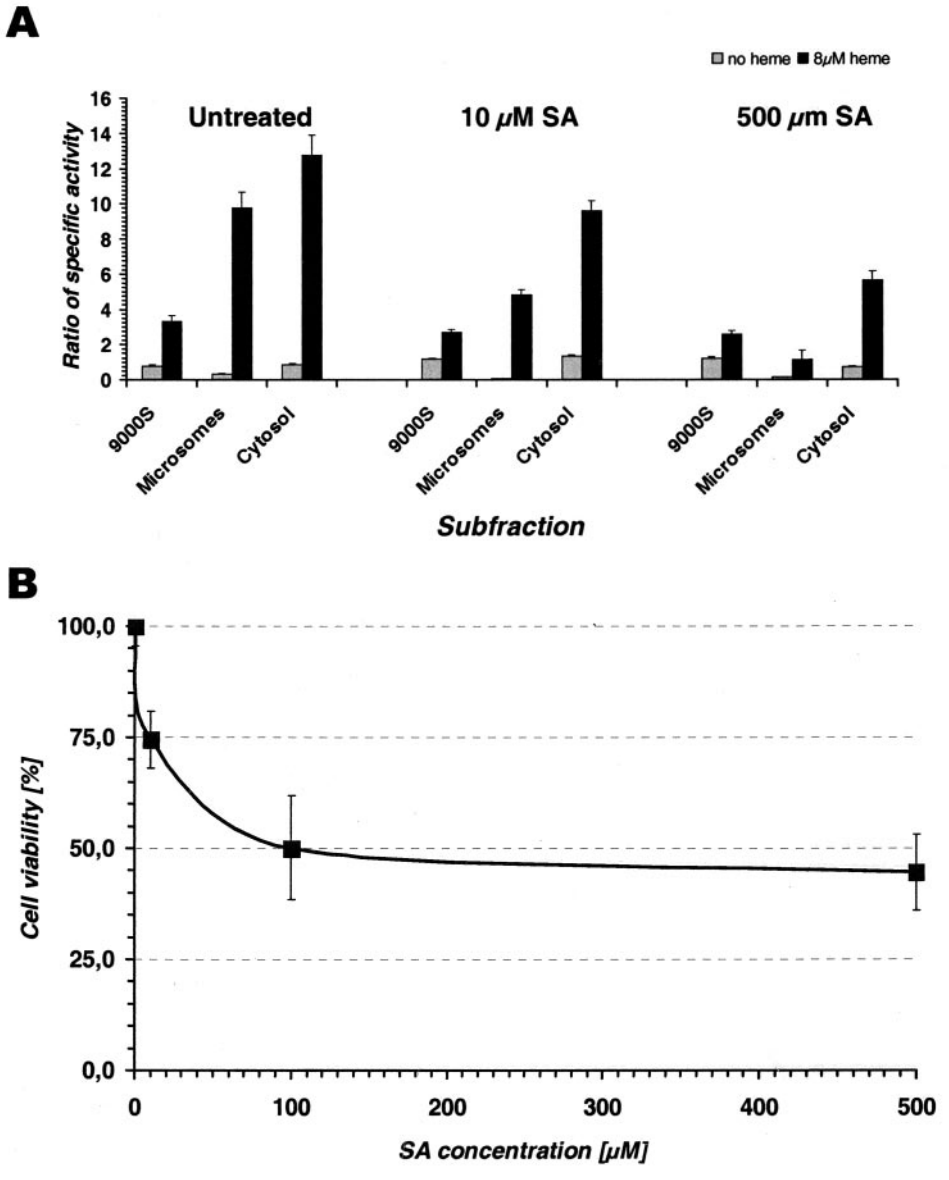

Fig. 3. A, EROD-activity in subfractions of COS-1 cells that were transiently transfected with CYP1A1 cDNA and cultivated in the presence of succinylacetone. The intended COS-1 cell subfractions (50–100 μg of protein) were incubated with 6 μM 7-ethoxyresorufin for 2 h at 37°C as described under *Materials and Methods*. Hematin (8 μM) was added to the samples where indicated. Activity is expressed as the ratio of specific EROD activity (picomoles per minute per milligram of protein) of *Cyp1a1*-plasmid transfected cells to activity of cells transfected with empty vector. Values represent mean ± S.E.M. of three independent experiments ($n = 3$). B, cell viability of COS-1 cells treated with SA (0, 10, 100, and 500 μM) for 48 h. Cells (2.5×10^4) in a single well of 96-well plates were investigated using the XTT method. OD-values from nontreated cells were set to 100%. Values represent mean ± S.E.M. ($n = 8$).

cell viability of about 25 (10 μM SA) to 55% (500 μM) (Fig. 3B). However, in COS-1 cells EROD-activity was quite low and only measurable after hematin addition (8 μM). CYP1A1 immunoblots of transfected COS-1 cells confirmed our findings analyzing EROD, because only in SA-treated cells was a cytosolic signal found (Fig. 4). Untreated COS-1 cells showed an immunosignal mainly in microsomes; cytosolic CYP1A1 expression was beyond the detection limit. These data suggest that incorporation of CYP1A1 into ER-membranes is impaired under heme-deficient conditions.

Discussion

In this report, we investigated the activation of CYP1A1 activity by hematin in mouse kidney and brain. Activation of monooxygenase activities by hematin has previously been reported in homogenates of rat, rabbit, and chicken extrahepatic tissues by Omiecinski et al. (1978). We have confirmed and extended theses findings. We first observed that the extrahepatic CYP1A1 occurred predominantly in the cytosol and that only its cytosolic enzymatic activity, measured as 7-ethoxyresorufin-deethylation, could be enhanced by addition of hematin (8 μM), presumably by reconstitution of apocytochrome. Soluble P450s are known to be present in bacteria (de Groot et al., 1996) and yeast (Yang et al., 1997). Mammalian P450s also can occur in soluble form when truncated at the N-terminal membrane anchor, although with impaired biological activity (Kempf et al., 1995). Interestingly, these soluble P450s, such as the one described here, did not distinguish between NADPH or NADH as cofactors. Other soluble enzymes such as NAD(P)H:quinone oxidoreductase$_1$ (NQO_1; DT-diaphorase) and myeloperoxidase, also use NADPH and NADH as cofactors and are able to metabolize 7-ethoxyresorufin (Joseph and Jaiswal, 1994; Pinnix et al., 1994; Neunaber and Achazi, 1999). Nevertheless, the contribution of these enzymes to the investigated CYP1A1-mediated EROD-activity and heme activation seems to be negligible. DT-diaphorase is unlikely to be enhanced by heme because of its isoalloxazine-ring system as prosthetic group (Poinas et al., 2002). The inhibition of EROD-activity by α-NF and CYP1A1-antibodies occurred both with heme exposure and without and therefore clearly indicates CYP1A1 activity (Fig. 1). Myeloperoxidase was excluded as contributor by gel-filtration chromatography (Taylor et al., 1990) (data not shown).

We next analyzed the causes for the localization of a nontruncated mammalian P450 in the cytosol. For this purpose, we expressed CYP1A1 in COS-1 cells derived from monkey kidney. Cytosolic CYP1A1 expression in COS-1 cells was inversely correlated with the availability of heme as deduced from experiments with SA, an inhibitor of heme biosynthesis

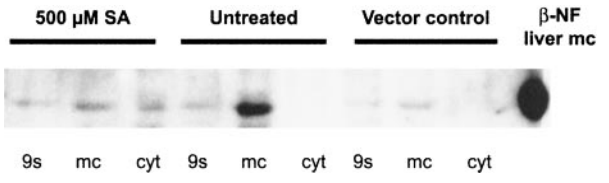

Fig. 4. Subcellular localization of expressed CYP1A1 in COS-1 cells. 0.1 mg of protein from 9000*g* supernatant (9s), microsomes (mc), and cytosol (cyt) was separated by SDS-PAGE. Immunoblot was performed with monoclonal antibody mab 1-7-1 raised against CYP1A1. Immunosignals were visualized using ECL.

(Giger and Meyer, 1983). These data suggest a decrease of microsomal incorporation of CYP1A1 when heme availability is limited. CYP1A1 immunoblots confirmed these findings as cytosolic CYP1A1 was found only in SA-treated COS-1 cells (Fig. 4). Succinylacetone at the concentration used to inhibit heme synthesis unfortunately also has some toxic effects on cells [see e.g., Vijayasarathy et al., (1999)]. Thus, the EROD activities are lower in SA-treated cells compared with controls, which is in accordance with cell viability experiments (Fig. 3). Nevertheless, the dose-dependent effect of SA on the ratio of cytosolic to microsomal EROD on heme-treated COS-1 cells is consistent with the results obtained in mouse brain.

The analysis revealing diminished heme content in extrahepatic tissue compared with liver (Table 2) supports our findings. The heme content of brain olfactory bulb and kidney (picomoles of heme per milligram of tissue) is only 39.6 and 37.7% of liver heme, respectively. Both tissues exert high levels of heme turnover and of heme-degrading enzyme heme oxygenase to generate second messenger carbon monoxide, which leads to a further diminution of free accessible heme (Ingi et al., 1996) (da Silva et al., 2001).

We conclude that the availability of heme is a limiting factor of P450 function in extrahepatic tissue. Moreover, heme seems to affect the incorporation of CYP1A1 into the ER membrane. The process of insertion of heme into the apocytochromes P450 and the mechanism of incorporation into the ER are incompletely known. We speculate that reduced availability of heme may limit the incorporation of P450s into ER membranes and therefore impair their function. The CYP1A1 apoprotein may occur as genuine cytoplasmic protein or, alternatively, a protein attached only weakly to ER-membranes—and therefore highly dissociable when homogenizing cells or tissue. This question is under current investigation using green fluorescent protein-labeled proteins and confocal microscopy.

P450 function is disturbed in situations of impaired heme synthesis such as acute hepatic porphyrias (Bonkovsky et al., 1991). When these patients are treated with heme infusion, their P450-dependent drug metabolism markedly increases, as determined by antipyrine clearance. Furthermore, heme infusions to healthy volunteers also increase P450-dependent antipyrine clearance. These clinical observations support our experimental data and suggest that also in human tissues apocytochrome P450 may not be saturated with heme. Therefore it is of particular interest, if the heme effect is also subject to other P450s than CYP1a1. Studies on CYP2C29 and CYP3A-isoforms, which are markedly expressed in brain, are presently initiated in our laboratory.

The demonstration of high levels of heme-activated EROD in brain olfactory bulb, but lower activities in hypothalamus, striatum and cortex was unexpected, as all these tissues express CYP1A1 mRNA (Schilter and Omiecinski, 1993). Several P450s, including CYP1A, 1B, 2A, 2G, are highly expressed in the olfactory mucosa at levels comparable with liver (Gu et al., 1998; Huang et al., 2000). These enzymes presumably play an important and unique role in the detoxification of xenobiotics (drugs, odorants) entering the body through the nose (Reed et al., 1988). If the olfactory bulb represents a second barrier of defense against foreign compounds is an interesting question.

In conclusion, the results of the present study suggest a

mechanism by which extrahepatic tissues regulate P450 function. The availability of heme seems to be a crucial factor for these activities. Our observations therefore are of relevance for the assessment of the physiologic function of brain P450s in the metabolism of drugs and other compounds.

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