

Cytochrome P450 1B1 mRNA in the human central nervous system

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Abstract

Aims—To study the expression of CYP1B1 in a variety of human and rat cell lines as a means of identifying a new tool for the investigation of gene regulation. In addition, to identify the expression of cytochrome P450 1B1 (CYP1B1) in different regions of the central nervous system (CNS).

Methods—Reverse transcription-polymerase chain reaction followed by cloning and sequencing were used to detect the expression of CYP1B1 in human cell lines. Poly A+ mRNA from the human spinal cord and from different brain regions was analysed using a CYP1B1 probe labelled with ³²PdCTP.

Results—Expression of CYP1B1 was shown in a human astrocytoma cell line (MOG-G-CCM). CYP1B1 mRNA was expressed in a variety of regions of the CNS but with a distinct regional specificity. Expression was highest in the putamen.

Conclusions—The expression of CYP1B1 in a human astrocytoma enables this cell line to be used in further studies of regulation and function of this gene. The demonstration that CYP1B1 mRNA is expressed in a variety of regions of the CNS suggests a role for this gene in brain and spinal cord metabolism. The regional specificity of expression might explain the focal damage of certain human neurodegenerative diseases.

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The P450 genes are a superfamily of genes encoding an array of haem containing enzymes that play an important role in the biotransformation of many endogenous and exogenous compounds.¹⁻⁴ Cytochrome P450 enzymes represent one of the first lines of defence against toxic foreign compounds, including drugs and environmental chemicals. In general, cytochrome P450s mediate detoxification reactions; however, under certain circumstances they can activate their substrates to form carcinogenic, mutagenic, and cytotoxic products.¹⁻⁶

Although quantitatively the liver is the primary organ involved in P450 mediated metabolism, increasingly, the importance of extrahepatic P450 mediated metabolism is being recognised, especially with respect to its potential role in extrahepatic target organ toxicity.⁷⁻⁹ The central nervous system (CNS)

might be the target for the pharmacological and/or toxicological effects of a wide variety of lipophilic drugs and chemicals present in the environment. If these lipophilic compounds are not biotransformed to easily excretable metabolites, they might accumulate in lipid rich tissues, such as the brain. In view of this, bioactivation and detoxification of environmental toxins by cerebral P450 might be important.⁷⁻¹¹ In addition to the ability to metabolise drugs, there is evidence for the involvement of brain cytochrome in the generation of active oxygen radicals and lipid peroxides.¹²

Exposure to environmental or endogenous toxins in genetically predisposed individuals might have an important role in the aetiopathogenesis of neurodegenerative disorders. Parkinson's disease has been associated with mutations in the CYP2D6¹³⁻¹⁵ and CYP1A1 genes.¹⁶ Some hepatic cytochrome P450 enzymes such as CYP2D6,¹⁷⁻¹⁸ CYP1A2,¹⁷ CYP3A4,¹⁷ and CYP2C11¹⁹ have been shown to be involved in the metabolism of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPTP induces a Parkinson-like syndrome through biotransformation to the neurotoxic metabolite 1-methyl-4-phenylpyridinium (MPP⁺). It has been shown that human cytochrome P450 enzymes catalyse the N-demethylation of MPTP. The hepatic N-demethylation of MPTP metabolises MPTP to PTP (4-phenyl-1,2,3,6-tetrahydropyridine).¹⁸ Unlike MPTP, PTP has been shown to be relatively non-toxic, both in vitro and in vivo, suggesting that cytochrome P450 might be a pathway of detoxification.¹⁸ In contrast, a possible involvement of cytochrome P450 in MPTP induced neurotoxicity has also been suggested.²⁰⁻²¹ Neurotoxicity induced by MPTP in mouse brain slices was prevented by prior exposure of the slices to cytochrome P450 inhibitors (piperonyl butoxide and SKF 525A), and MPTP toxicity was potentiated significantly in mouse brain slices prepared from mice pretreated with phenobarbital, an inducer of cytochrome P450 (P450 2B1 and P450 2B2).²⁰ In view of this, the cerebral P450 mediated metabolism leading to bioactivation or detoxification of toxins in situ in the brain would be of great importance.

P450 enzymes and associated mono-oxygenase activities are present in the brain tissue of rodents,^{11 22-25} monkeys,²⁶ and humans.²⁷⁻²⁹ The concentrations of P450 and associated xenobiotic mono-oxygenase activities have been studied in different regions of the brain.²⁷⁻²⁹ In the human brain, the highest concentrations of P450 for each gram of tissue and associated mono-oxygenase activity have

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been found in the brainstem and cerebellum and the lowest were found in the striatum and hippocampus.^{27, 28} The regional expression of CYPs in the human brain might explain the focal CNS damage of certain neurodegenerative diseases.

During recent years, studies have revealed regional heterogeneity in the distribution of specific P450 forms in rodents^{30, 31} and humans.³² The 1A subfamily is one of the most abundant forms of xenobiotic enzymes in the human CNS.³⁰ It is detected in the striatum, hypothalamus, cerebellum, medulla oblongata, olfactory bulbs, midbrain, and cortex.³⁰ However, there is still considerable controversy over the expression and localisation of these enzymes in the brain.

CYP1B1, recently identified, is the newest member of human cytochrome P450 family 1 and is unique among known P450 cytochromes in exhibiting both hormonal regulation and stimulation by AhR (aryl hydrocarbons receptors) ligands in a highly tissue specific manner.³³⁻³⁵ It has been demonstrated that CYP1B1 is involved in the metabolism of xenobiotics and is an important enzyme in the activation of a variety of environmental carcinogens and mutagens.³⁶ In humans, the CYP1B1 gene is expressed constitutively, mainly in extrahepatic organs.^{36, 37} It is expressed constitutively in the adrenals, ovary, and testis, where it is inducible by polycyclic aromatic hydrocarbons, adrenocorticotropin, and other peptide hormones.^{33, 36, 38} Therefore, P4501B1 represents an extrahepatic route of xenobiotic metabolism and of hormonal regulation.^{36, 38-40}

There have been few reports of localised gene expression of individual CYPs within the human brain.^{18, 32} CYP1B1 expression was detected in the normal human brain by northern blotting,^{33, 36} but no studies of CYP1B1 and brain region distribution have yet been performed.

The present study was carried out to find a cell line of CNS origin that expressed CYP1B1 for two reasons: to provide a tool for studying gene regulation and to identify regional expression of CYP1B1 in the CNS. Two human glioma cell lines (MOG-G-CCM and A172) and a rat pheochromocytoma cell line (PC12) were studied. Although PC12 cells are not derived from the CNS, they have been used frequently to study CNS related functions.^{41, 42} At the same time HepG2 (human hepatocellular carcinoma cell line) was investigated as a possible tool because of the expression of CYP1B1 in normal human liver. Human pheochromocytoma and rat adrenal tissues were used as positive controls in the reverse transcription-polymerase chain reaction (RT-PCR) reactions. CYP1B1 mRNA expression in various regions of normal human brain and in the spinal cord was analysed by northern blotting.

Methods

CELLS AND CULTURE CONDITIONS

Human astrocytoma (MOG-G-CCM), glioblastoma (A172), PC12, and HepG2 cell lines

were obtained from the European Collection of Animal Cell Cultures, Porton Down, Salisbury, UK. MOG-G-CCM and A172 were cultured in DMEM supplemented with 10% fetal calf serum (FCS). HepG2 cells were grown in RPMI 1640 supplemented with 10% FCS. In addition, both culture media contained L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 mg/ml). PC12 cells maintained in suspension (proliferating pheochromocytoma cell-like phenotype) were cultured in RPMI 1640 medium supplement with 10% horse serum, 5% FCS, L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 mg/ml). Differentiation of PC12 cells into a non-proliferating, neurite bearing, sympathetic-like neuron phenotype was obtained by maintaining the cells in tissue culture flasks prepared with collagen type 1 and adding nerve growth factor (NGF) to the culture media (50 ng/ml) as described elsewhere.⁴² These cells were also treated with β -naphthoflavone, a CYP1B1 inducer. All the cell culture reagents were purchased from Sigma Chemical Co (Poole, Dorset, UK).

TISSUE SAMPLES

A sample of human pheochromocytoma was excised from a female patient undergoing surgery for the removal of a large adrenal tumour. Male rat adrenal tissue was obtained from the Biomedical Services Unit, University of Birmingham. In both instances, tissue was snap frozen in liquid nitrogen within a few minutes of removal.

RNA AND CDNA

Total RNA was isolated from the cell lines and tissues using RNeasy reagent (AMS Biotechnology, Benelux, UK), as directed in the manufacturer's protocol. cDNA probes for CYP1B1 were prepared by reverse transcription-polymerase chain reaction (RT-PCR) amplification of total MOG-G-CCM RNA. The human CYP1B1 primers designed were: 5'-CCA CGA CGA CCC CGA GTT CC-3' (forward) and 5'-GAG ACC AAT CTT GGA TTC CCA CC-3' (reverse). They were predicted to give a product of 1387 base pairs and were directed against nucleotide positions 991-2355 of human CYP1B1 cDNA. Between the upper and lower primers, there was one intron to differentiate the RT-PCR product from that produced by genomic DNA amplification. PCR using a hot start technique was performed in a 50 μ l final volume consisting of 1 μ l reverse transcribed product, 5 μ l Taq 10 \times NH₄ buffer, 5 μ l 2.5 mM dNTPs, 5 μ l 10 μ M CYP1B1 forward primer, 5 μ l 10 μ M CYP1B1 reverse primer, 1.5 μ l 50 mM MgCl₂, 27 μ l Milli-Q water, and 0.5 μ l Taq polymerase (Promega, Southampton, UK). The thermocycler conditions used were the following: 93°C for 30 seconds (denaturation), 60°C for 30 seconds (annealing), and 72°C for 20 seconds (extension) performed for 35 cycles.

The PCR products were cloned into the pMOSBlue T vector (Amersham-Life Science, Amersham, UK) and the identity was confirmed by automated cycle sequencing (Alta

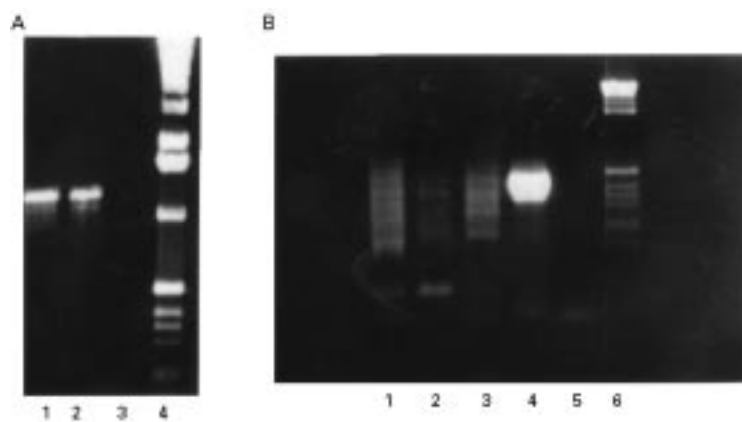


Figure 1 Polymerase chain reaction amplified CYP1B1 in HepG2, MOG-G-CCM, and PC12 cell lines. Primer sequences are provided in the methods section. (A) CYP1B1 expression in: HepG2 cells, lane 1; MOG-G-CCM, lane 2; and negative control, lane 3. (B) CYP1B1 expression in: PC12 pheochromocytoma cell line phenotype, lane 1; PC12 neuron phenotype, lane 2; PC12 neuron phenotype cell line treated with β -naphthoflavone, lane 3; normal rat adrenal, lane 4; and negative control, lane 5.

Bioscience, Birmingham University, UK). To exclude the chances of cross-hybridisation with other sequences, the sequence was compared with the Genbank using the Genetics Computer Group FASTA program. Primers for the rat CYP1B1 were: 5'-GCT GTA TGC TTC GGC TGT CGG-3' (forward) and 5'-CAA CCT GGT CCA ACT CGG CC-3' (reverse). They were in positions 990 and 1442, respectively, of the rat CYP1B1 cDNA and were predicted to yield a 472 base pair (bp) product. RNA from normal rat adrenal gland was used as a positive control for RT-PCR in PC12 cells.

BRAIN TISSUE SAMPLES AND NORTHERN BLOT ANALYSIS

Poly A+ mRNA brain tissue blots were obtained from Clontech Laboratories (Palo Alto, California, USA; 7750-1 Human Brain MTN Blot III and 7755-1 Human Brain MTN Blot II). According to the manufacturer's information, each lane of a multiple tissue northern blot contains ~ 2 μ g of purified poly A+ mRNA from different brain regions that had run on a denaturing formaldehyde/1.2% agarose gel and had been blotted on to a positively charged nylon membrane. The blots were prehybridised in 5 \times SSC (saline sodium citrate), 5% dextran sulphate, 5 \times Denhardt's solution, and 100 μ g/ml heat denatured herring sperm DNA at 65°C for two hours. Hybridisation was performed at 65°C overnight using human cDNA probes for CYP1B1. Probes were labelled with α^{32} PdCTP by random hexanucleotide priming using the Prime-a-Gene labelling system (Promega). The membrane was rinsed with 2 \times SSC at room temperature and then washed with 2 \times SSC, 0.1% SDS (sodium dodecyl sulphate) at 55°C for 20 minutes. The washing solution was discarded and the procedure was repeated using progressively lower concentrations of SSC (1 \times , 0.5 \times , and 0.1 \times). After washing, the membranes were placed on Kodak BioMax MS film with intensifying screens for 120 hours at -70°C. Subsequently, the membranes were stripped and reprobbed with a 2.0 kilobase (kb) human

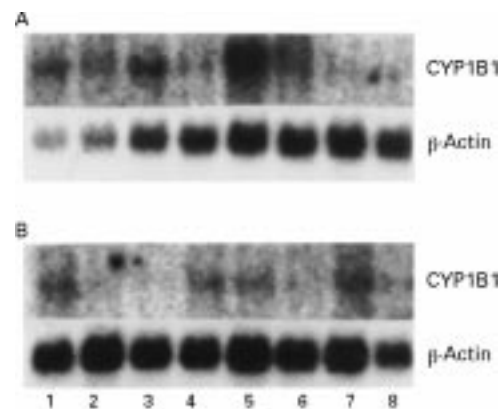


Figure 2 Expression of the CYP1B1 gene in brain regions and the spinal cord. mRNA blots were hybridised successively with CYP1B1 and β -actin cDNA. The specific mRNAs are identified at the right. (A) Blot 1: putamen, lane 1; temporal lobe, lane 2; frontal lobe, lane 3; occipital lobe, lane 4; spinal cord, lane 5; medulla, lane 6; cerebral cortex, lane 7; cerebellum, lane 8. (B) Blot 2: thalamus, lane 1; subthalamic nucleus, lane 2; substantia nigra, lane 3; whole brain, lane 4; hippocampus, lane 5; corpus callosum, lane 6; caudate, lane 7; amygdala, lane 8.

Table 1 Regional CYP1B1 and β -actin mRNA concentrations and ratio based on densitometric analysis

| Region | CYP1B1 | β -Actin | Ratio |
|---------------------|--------|----------------|-------|
| Putamen | 1.125 | 0.607 | 1.853 |
| Spinal cord | 4.658 | 3.081 | 1.512 |
| Medulla | 2.996 | 3.001 | 0.998 |
| Temporal lobe | 0.984 | 0.993 | 0.991 |
| Frontal lobe | 1.855 | 2.029 | 0.914 |
| Occipital lobe | 0.893 | 2.293 | 0.389 |
| Cerebellum | 0.410 | 2.597 | 0.158 |
| Cerebellum cortex | 0.571 | 3.712 | 0.154 |
| Thalamus | 1.052 | 3.740 | 0.281 |
| Whole brain | 1.020 | 4.152 | 0.246 |
| Amygdala | 0.493 | 2.297 | 0.215 |
| Caudate | 0.932 | 5.488 | 0.170 |
| Corpus callosum | 0.770 | 4.567 | 0.169 |
| Hippocampus | 1.060 | 6.589 | 0.160 |
| Subthalamic nucleus | 0.450 | 6.041 | 0.074 |
| Substantia nigra | 0.261 | 4.290 | 0.061 |

β -actin cDNA probe. The mRNA levels were determined by densitometric analysis of the film exposed to the CYP1B1 and β -actin probes.

Results

CYP1B1 expression was detected by RT-PCR in astrocytoma cell line (MOG-G-CCM). PCR amplification produced a band of the expected size (~ 1.4 kb) visible by agarose gel analysis. No additional bands were amplified, and the presence of one intron between the upper and lower primers differentiated it from DNA contamination. Sequencing of the cloned PCR products confirmed identity with CYP1B1. CYP1B1 expression was not detected in glioblastoma cell line (A172). No expression was seen in either the PC12 cell line (pheochromocytoma cell-like and neuron-like phenotype) even when treated with β -naphthoflavone. HepG2 cells did express readily detectable mRNA (fig 1). CYP1B1 expression was also detected in a human pheochromocytoma tissue and in normal rat adrenal tissue.

Figure 2 shows northern blot analysis of CYP1B1 expression in different brain regions and spinal cord. CYP1B1 gene expression was detected in most of the regions analysed. The

most intense hybridisation signals (highest CYP1B1:β-actin ratio) occurred in the spinal cord and putamen (table 2). Apart from the putamen, CYP1B1 gene expression in the medulla oblongata and in the temporal and frontal lobes appeared greater than in the others regions of the brain. Lower expression was also detected in the thalamus, hippocampus, corpus callosum, caudate, amygdala, temporal lobe, and occipital lobe. Expression in the subthalamic nucleus, substantia nigra, cortex, and cerebellum was barely detectable.

Discussion

Our study reports CYP1B1 expression in a brain tumour cell line (MOG-G-CCM). The presence of CYP1B1 protein in astrocytoma cells was confirmed recently by immunohistochemistry.⁴³ Although tumour cells might regulate the expression of these genes differently from normal cells, the demonstration of CYP1B1 expression in human astrocytoma cells enables the use of this cell line in further studies of regulation and function of this gene. It has also been suggested that the expression of CYP1B1 in malignant tumours might have important consequences for the diagnosis and treatment of cancer.⁴³

Our study reports for the first time the localisation of CYP1B1 mRNA expression in the human CNS. The northern blot results obtained in our study indicate that CYP1B1 mRNA is expressed with regional specificity in the CNS. Of the different regions analysed, the highest concentrations of CYP1B1 mRNA were seen in the putamen and spinal cord tissue.

Recently, cytochrome P450 and associated mono-oxygenase activities were demonstrated in the human spinal cord.²³ Using immunoblot analysis, cytochrome P450 enzymes, namely 1A1, 1A2, 2E1, and 2B1 have been reported in the rat and human spinal cord. However, none of these forms showed higher mono-oxygenase activity than that seen in whole brain.²³ The presence of cytochrome P450 in the spinal cord suggests that the spinal cord can metabolise xenobiotics and detoxify them effectively or bioactivate an inert, non-toxic compound to a reactive, electrophilic metabolite, which can interact extensively with cellular constituents, leading to cellular damage or cell death.²³

The CYP1B1 mRNA concentrations shown in the current study indicate also that the CYP1B1 gene is expressed with distinct regional specificity in the human brain. In this respect, the results indicated higher expression of CYP1B1 mRNA in the putamen when compared with other regions. Although regional gene expression of CYP1B1 has not been published, regional expression of other individual CYPs in the human brain has been reported.³² Similarly to CYP1B1, other cytochrome P450 enzymes (CYP1A1, CYP1A2, CYP2E1, and CYP3A) were also evident in most brain regions examined.³² In contrast to CYP1A1, which showed a high level of expression in the cerebellum,³² in the present study, CYP1B1 mRNA expression was very low in this region. In another study in the rat, the cerebellum

CYP2B subfamily showed relatively high expression, whereas transcripts of the 1A subfamily were found only at low concentrations.³⁰ In the human brain, the CYP1A2 mRNA concentrations were highest in the frontal and occipital lobes, whereas the red nucleus and substantia nigra had lower concentrations compared with the other brain regions surveyed. CYP2E1 gene expression in the cerebellum, frontal lobe, occipital lobe, and pons region was similar, but appeared greater than in the red nucleus and substantia nigra. CYP3A gene expression was most clearly detectable in the pons; lower concentrations of CYP3A mRNA were detected also in the cerebellum, frontal lobe, occipital lobe, and red nucleus. CYP3A mRNA was not detected in the substantia nigra.³² CYP2D6 mRNA was identified in the substantia nigra of the human adult brain using hybridisation *in situ*, although the CYP2D6 signal was much weaker than the β-actin control with respect to exposure time and probe length.¹⁸

In our study, the substantia nigra showed low CYP1B1 expression whereas the putamen showed the highest expression of CYP1B1 mRNA. Parkinson's disease is characterised by the degeneration of the dopamine neurons of the substantia nigra pars compacta. It has been suggested that the striatum, the major projection of the substantia nigra pars compacta, might be the primary target of neurotoxins and might be the first area to die, followed by the slower secondary death of the substantia nigra pars compacta dopamine cell bodies.⁴⁴ Consistent with this theory are the observations that MPP⁺ accumulates primarily in striatal dopamine terminals and not in the substantia nigra dopamine cell bodies after MPTP administration.⁴⁵ Intra-striatal injection of 6-hydroxydopamine, another dopamine neurotoxin, also destroys dopamine nerve terminals and leads to a retrograde degeneration of the substantia nigra pars compacta dopamine cell bodies.⁴⁴

It has been suggested that small amounts of enzyme distributed uniformly throughout the brain would offer no protective advantage to any area of the brain in terms of enhanced xenobiotic elimination from this tissue. However, the more localised the enzyme becomes, the greater its impact on the xenobiotic concentrations in those localised areas of the brain where it is expressed. It is possible that deficiencies in the expression of drug metabolising enzymes in the CNS might have as great, or greater, an effect on the xenobiotic response in the CNS than deficiencies in hepatic oxidative drug metabolism. This could be particularly harmful if a large amount of the xenobiotic reached the CNS because of an inability to undergo metabolism in the liver.⁴⁶

In summary, the expression of CYP1B1 mRNA in the spinal cord and in many brain regions was demonstrated, suggesting a role in local metabolism. This regionally specific expression of CYP1B1 in the human CNS might explain the focal CNS damage of certain neurodegenerative diseases.

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