Differential Expression of Human Cytochrome P450 Enzymes from the CYP3A Subfamily in the Brains of Alcoholic Subjects and Drug-Free Controls

Iris M. Booth Depaz, Francesca Toselli, Peter A. Wilce, and Elizabeth M. J. Gillam

School of Biomedical Sciences (I.M.B.D.), and School of Chemistry and Molecular Biosciences (F.T., P.A.W., E.M.J.G.), The University of Queensland, Brisbane, Queensland, Australia

Received February 10, 2013; accepted March 14, 2013

ABSTRACT

Cytochrome P450 enzymes are responsible for the metabolism of most commonly used drugs. Among these enzymes, CYP3A forms mediate the clearance of around 40–50% of drugs and may also play roles in the biotransformation of endogenous compounds. CYP3A forms are expressed both in the liver and extrahepatically. However, little is known about the expression of CYP3A proteins in specific regions of the human brain. In this study, form-selective antibodies raised to CYP3A4 and CYP3A5 were used to characterize the expression of these forms in the human brain. Both CYP3A4 and CYP3A5 immunoreactivity were found to vary among regions in the microsomal fractions of cortex, hippocampus, basal ganglia, amygdala, and cerebellum. However, only CYP3A4 expression was observed in the mitochondrial fractions of these brain regions. N-terminal sequencing confirmed the principal antigen detected by the anti-CYP3A4 antibody in cortical microsomes to be CYP3A4. Immunohistochemical analysis revealed that CYP3A4 and CYP3A5 expression was primarily localized in the soma and axonal hillock of neurons and varied according to cell type and cell layer within brain regions. Finally, analysis of the frontal cortex of chronic alcohol abusers revealed elevated expression of CYP3A4 in microsomal but not mitochondrial fractions; CYP3A5 expression was unchanged. The site-specific expression of CYP3A4 and CYP3A5 in the human brain may have implications for the role of these enzymes in both normal brain physiology and the response to drugs.

Introduction

Cytochrome P450 (P450) enzymes are monoxygenases that catalyze the oxidative metabolism of a great variety of xenobiotic and endogenous chemicals such as drugs, environmental contaminants, steroids, and fatty acids. P450s of families CYP1–CYP3 show particularly wide and overlapping substrate ranges, a property that enables them to mediate approximately 75% of the metabolic clearance of drugs in humans (Wienkers and Heath, 2005). Most of the drug-metabolizing forms are expressed at high levels in the liver, but many also show a widespread pattern of extrahepatic distribution (Guengerich, 2005). Several members of families CYP1–CYP3 have been reported in human brain [reviewed in Meyer et al. (2007) and Dutheil et al. (2010)] and the importance of these P450s in the metabolism of endogenous compounds in the brain and their potential role in normal neurologic function and disease have been highlighted recently (Strobel et al., 2001; Haining and Nichols-Haining, 2007; Meyer et al., 2007; Niwa et al., 2009).

This research was supported by the National Health and Medical Research Council [Grant 210215 to P.A.W. and E.M.J.G.]. This work was previously presented in preliminary form at the following meeting: Depaz IM, Wilce PA and Gillam EMJ (2006) Detection of specific members of the cytochrome P450 2C and 3A subfamilies in the human brain. Fourteenth North American International Society for the Study of Xenobiotics Meeting; 2006 Oct 22–26; Rio Grande, Puerto Rico. dx.doi.org/10.1124/dmd.113.051359.

This article has supplemental material available at dmd.aspetjournals.org.

ABBREVIATIONS: I.I., integrated intensity; P450, cytochrome P450, heme-thiolate protein P450; PBS, phosphate-buffered saline; PVDF, polyvinylidene fluoride.
detected in dura mater, cortex, cerebellum, basal ganglia (nucleus accumbens and putamen), midbrain (red nucleus), pons, hippocampus, medulla, postcentral and paracentral gyrus, corpus callosum, insula (McFadyen et al., 1998; Agarwal et al., 2008; Dutheil et al., 2009), and anterior pituitary (Murray et al., 1995). Finally, CYP3A43 mRNA has also been reported in human brain, where it seems to localize to the cortex, cerebellum, basal ganglia (nucleus accumbens), midbrain (reticular neurons and red nucleus), pons, hippocampus, dentate gyrus, medulla, spinal cord, and dura mater (Agarwal et al., 2008; Dutheil et al., 2009). Interestingly, its expression in human brain seems to be higher than in liver from the same individual, and equal to or higher than brain CYP3A4 (Agarwal et al., 2008).

Studies have also suggested that brain CYP3A forms are capable of metabolizing xenobiotic compounds (Voirol et al., 2000; Pai et al., 2002; Agarwal et al., 2008; Ghosh et al., 2010); however, data on specific protein expression are limited (Ghosh et al., 2011). Detection of individual CYP3A proteins in the brain has been hindered by a lack of antibodies directed to specific subfamily members, as well as limited access to adequate amounts of human brain tissue.

The objective of the current study was to elucidate the pattern of CYP3A4 and CYP3A5 protein expression in five regions of the human brain (frontal cortex, hippocampus, basal ganglia, amygdala, and cerebellum) and to assess the relative expression of CYP3A4 and CYP3A5 in alcoholic subjects.

Materials and Methods

Materials. Polyvinylidene fluoride (PVDF) membrane and BioTrace NT nitrocellulose membranes were obtained from Pall Corporation (East Hills, NY). A number of commercially available antibodies were used in this study. Mouse anti-human-α-tubulin monoclonal primary antibody was purchased from Sigma-Aldrich (St. Louis, MO). The secondary antibodies used for human brain tissue immunoblots were AlexaFluor 680-labeled goat anti-rabbit IgG antibody (Invitrogen, Carlsbad, CA) and IRDye 800-labeled donkey anti-mouse IgG antibody (Rockland Immunochimicals, Gilbertsville, PA). The Odyssey infrared system was used for immunoblot imaging and analysis (LI-COR, Lincoln, NE). An AlexaFluor 488-labeled goat anti-rabbit IgG secondary antibody was used for immunohistochemistry (Invitrogen). Fluorescence mounting medium (DAKO, Glostrup, Denmark) was used to maintain fluorophore stability.

Antibody Production. CYP3A4- and CYP3A5-specific antibodies were generated utilizing bacterially expressed recombinant proteins, expressed and purified as previously described (Gillam et al., 1993, 1995). Preimmune serum (20 ml) was collected from two 12-week-old New Zealand white rabbits and then purified, and recombinant CYP3A4 or CYP3A5 (100 μg) was injected into each animal. Three subsequent boosters of 50 μg each were administered fortnightly thereafter. Animals were exsanguinated 11 weeks after initial immunization and serum was prepared as previously described (Depaz et al., 2003). IgG fractions were then prepared by sodium sulfate precipitation (Harlow and Lane, 1999), followed by desalting on a PD-10 desalting column (GE Healthcare, Rydalmere, Australia) and elution in phosphate-buffered saline (PBS) containing 0.02% (w/v) sodium azide. Antibody aliquots (approximately 200 μl) were snap frozen in liquid nitrogen and stored at −80°C.

To further enhance specificity, antibodies were isolated from immunoblots as previously described (Worrall et al., 1993). Briefly, bacterial membrane fractions containing 1800 pmol recombinant CYP3A4 or 1400 pmol recombinant CYP3A5 were electrophoresed on SDS-polyacrylamide gels (4% stacking gel and 10% resolving gel) then transferred onto a PVDF membrane (Towbin et al., 1979). Membranes were blocked with 1.35% (w/v) fish gelatin in PBS for 1 hour at room temperature, and then incubated with primary antibody overnight at 4°C at a dilution of 1:1000 in 1.35% (w/v) fish gelatin in PBS. Membranes were then washed and incubated with secondary Alexa 680-labeled goat anti-rabbit antibody (1:20,000) for 30 minutes at 25°C, before the washing and visualization on the Odyssey imaging system. The specific CYP3A4 or CYP3A5 band was excised from the membrane and the primary antibody was eluted with 2 ml elution buffer [200 mM glycine, 0.2% (w/v) gelatin, pH 2.8] for 2 minutes at 37°C. The eluate was neutralized with an equal volume of 1 M Tris base (no pH adjustment) and the resulting affinity-purified antibody preparation was used immediately in further immunoblottting experiments. Affinity-purified antibodies raised against CYP3A4 or CYP3A5 were tested for specificity to all four recombinant human CYP3A proteins (CYP3A4, CYP3A5, CYP3A7, and CYP3A43) expressed in bacterial membrane fractions. Equimolar amounts of each recombinant enzyme (0.5 pmol spectrally detectable P450) were subjected to SDS-PAGE, and transferred to PVDF membranes as described above. Blots were incubated with affinity-purified primary anti-P450 antibodies at a dilution of 1:10 in 1.35% (w/v) fish gelatin in PBS and incubated on membranes overnight at 4°C. Incubation with AlexaFluor 680-labeled goat anti-rabbit IgG secondary antibody and fluorescence detection was performed as described above. For each antibody, the cross-reactivity for individual P450 forms was expressed as the ratio of integrated intensity (I.I.) for the P450 in question to that of the target CYP3A (I.I._CYP/I.I.CYP3A4/5).

Preparation of Mitochondrial and Microsomal Protein Fractions. Frozen human brain samples were obtained from the New South Wales Tissue Resource Centre (The University of Sydney, NSW, Australia). For the first part of the study in which the subcellular localization of CYP3A4 and CYP3A5 was examined in five brain regions [frontal cortex (Brodmann area 9), anterior hippocampus, basal ganglia, amygdala, and cerebellum], samples from three different male subjects (a total of 15 tissue samples) were provided as dissected sections. The samples were taken from the right hemisphere of alcohol-free and illicit drug–free patients. The histologic appearance was reported as normal at both the macroscopic and microscopic levels. Details of patient histories are provided in Table 1. The samples used to compare the expression of P450s in the alcoholic and nonalcoholic brain were obtained from 12 different male subjects, the details of which are listed in Table 2. Frozen brain tissue samples (approximately 500 mg) were placed into 5 ml ice-cold homogenization solution (1.14% w/v KCl and 10 mM EDTA, pH 7.5, with 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM dithiothreitol, and 0.1 mM butylated hydroxytoluene added immediately prior to homogenization). Samples were manually crushed with a Teflon homogenizer for no more than 45 seconds (approximately 10–12 repetitions) and then kept on ice prior to centrifugation at 3000g for 5 minutes at 4°C. The supernatant (S3000 fraction) was then centrifuged at 10,000g for 30 minutes at 4°C. The resulting supernatant (S10000 fraction) was separated from the pellet and centrifuged at 110,000g for 60 minutes at 4°C. Mitochondrial (10,000g) and microsomal (110,000g) pellets were then resuspended in a volume of ice-cold buffer (250 mM sucrose and 10 mM EDTA, pH 7.5) equivalent to 3.2- and 1.6-fold their wet weight, respectively. Aliquots (25 μl) of each fraction were snap frozen in liquid nitrogen and stored at −80°C.

Immunoblot Analysis. Three control brain samples (Table 1) were used for immunoblot analysis of CYP3A4 and CYP3A5 expression in different brain regions and subcellular fractions. Samples (20 μg protein per lane) were separated on SDS-PAGE and subjected to immunoblotting with

<table>
<thead>
<tr>
<th>TABLE 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient history details for control brain samples used for studies of the regio-selective expression of CYP3A proteins</td>
</tr>
<tr>
<td>Sample Code</td>
</tr>
<tr>
<td>Age (yr)</td>
</tr>
<tr>
<td>Sex</td>
</tr>
<tr>
<td>Postmortem delay (h)</td>
</tr>
<tr>
<td>Cause of death</td>
</tr>
</tbody>
</table>

Downloaded from dmd.aspetjournals.org on April 28, 2017
affinity-purified anti-CYP3A antibodies as described in the section on antibody production. Tissue samples were obtained from 12 different subjects for the analysis of CYP3A4 and CYP3A5 expression in the frontal cortex of alcoholic and nonalcoholic subjects (Table 2). Blots were prepared as described above and incubated with affinity-purified primary anti-P450 antibodies and AlexaFluor 680-labeled goat anti-rabbit IgG secondary antibody before analysis using the Odyssey system. Mouse anti-human-α-tubulin monoclonal primary antibody (used at a dilution of 1:20,000) was used as a loading control in these experiments and detected using an IRDye 800-labeled donkey anti-mouse IgG secondary antibody (1:20,000; Rockland Immunocemicals).

**Fluorescent Immunohistochemistry of Paraffin-Embedded Sections.** Slide-mounted 4-μm brain sections were first cleared of paraffin as previously described (Depaz et al., 2003), and then immersed in 10 mM sodium citrate buffer, pH 6.0, containing 0.05% (v/v) Tween-20, and heated to 97°C for 30 minutes. Sections were left to cool for 30 minutes and washed with PBS containing 0.05% (v/v) Tween-20 and rinsed in PBS before being treated with primary rabbit polyclonal P450 antibody (1:500) overnight at 4°C. Target proteins were eluted and samples were concentrated down to 800 μg total protein).

### TABLE 2

Clinical details and characteristics pertaining to alcoholic and control samples

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>Sex</th>
<th>Age</th>
<th>Postmortem Delay</th>
<th>Cause of Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>Male</td>
<td>50</td>
<td>17</td>
<td>Cardiac coronary artery atheroma</td>
</tr>
<tr>
<td>C2</td>
<td>Male</td>
<td>48</td>
<td>19</td>
<td>Coronary thrombosis</td>
</tr>
<tr>
<td>C3</td>
<td>Male</td>
<td>51</td>
<td>15</td>
<td>Pulmonary embolism</td>
</tr>
<tr>
<td>C4</td>
<td>Male</td>
<td>42</td>
<td>18</td>
<td>Suicide by hanging; asphyxia</td>
</tr>
<tr>
<td>C5</td>
<td>Male</td>
<td>45</td>
<td>16</td>
<td>Dilated cardiomyopathy</td>
</tr>
<tr>
<td>C6</td>
<td>Male</td>
<td>40</td>
<td>20</td>
<td>Ischemic heart disease</td>
</tr>
<tr>
<td>A1</td>
<td>Male</td>
<td>51</td>
<td>18</td>
<td>Alcohol toxicity</td>
</tr>
<tr>
<td>A2</td>
<td>Male</td>
<td>42</td>
<td>21</td>
<td>Ischemic heart disease</td>
</tr>
<tr>
<td>A3</td>
<td>Male</td>
<td>45</td>
<td>20</td>
<td>Consequences of cirrhosis</td>
</tr>
<tr>
<td>A4</td>
<td>Male</td>
<td>40</td>
<td>21</td>
<td>Acute alcohol poisoning</td>
</tr>
<tr>
<td>A5</td>
<td>Male</td>
<td>48</td>
<td>22</td>
<td>Bleeding esophageal varices</td>
</tr>
<tr>
<td>A6</td>
<td>Male</td>
<td>50</td>
<td>19</td>
<td>Cardiomyopathy</td>
</tr>
</tbody>
</table>

### Results

**Characterization of Antibody Selectivity.** When tested for specificity against recombinant CYP3A enzymes, affinity-purified anti-CYP3A4 and CYP3A5 antibodies showed cross-reactivity with other forms to different extents (Supplemental Fig. 1). For the antibody raised against CYP3A4, the cross-reactivity for each individual form was expressed as the ratio of integrated intensity for the P450 in question to that of CYP3A4 (L.I.\textsubscript{CYP3A4}), and was 0.51 and 0.93 for CYP3A5 and CYP3A7, respectively. For the antibody raised against CYP3A5, the cross-reactivity was expressed as the ratio L.I.\textsubscript{CYP3A5}, and was 0.51 and 0.81 for CYP3A4 and CYP3A7, respectively. No cross-reactivity was observed with CYP3A4 for either antibody under the high stringency conditions used, where blots were intentionally overloaded with recombinant antigens.

We cannot exclude the possibility that in immunoblotting and immunocytochemistry with brain samples the antibody raised against CYP3A4 is also detecting CYP3A7 and possibly CYP3A5, and that the antibody raised against CYP3A5 is also detecting CYP3A7 and possibly CYP3A4. However, two factors are important to note in the interpretation of these data. First, expression of CYP3A7 and CYP3A43 holoenzyme in Escherichia coli is poor relative to that of CYP3A4 and CYP3A5, meaning that for the same amount of holoenzyme, substantially greater amounts of CYP3A7 and CYP3A43 apoprotein were included in the comparison shown in Supplemental Fig. 1, which may have inflated the apparent cross-reactivity of each antibody toward CYP3A7. Second, the amount of recombinant enzyme used for the immunoblots in Supplemental Fig. 1 (0.5 pmol) exceeds many times the amounts seen in the native tissues; therefore, the chances of nonspecific cross-reactivity are increased. Moreover, different expression patterns were seen with each antibody suggesting preferential detection of two different antigens (Figs. 1 and 6) and N-terminal sequencing of proteins affinity-purified from cortical microsomes using the anti-CYP3A4 antibody showed that the predominant protein detected with this antibody in the cortex is CYP3A4 (see below).

**CYP3A Forms Are Differentially Expressed in the Human Brain.** Immunoblots using the affinity-purified polyclonal antibodies selectively targeted to CYP3A4 or CYP3A5 confirmed the presence of both CYP3A4 and CYP3A5 in all five brain regions analyzed, namely cortex, hippocampus, amygdala, basal ganglia, and cerebellum (Fig. 1). However, differential expression was observed between the cytosolic, mitochondrial, and microsomal fractions between CYP3A4 and CYP3A5. Although samples from only three individuals were available for detailed analysis, CYP3A expression patterns were consistent between samples with CYP3A signal detected in the same regional and subcellular locations. Expression levels varied slightly, with the sample with the longest postmortem delay having slightly lower levels of CYP3A. No signal was detected for any P450 protein in immunoblots with preimmune sera (unpublished data).

CYP3A4 was detected in the microsomes, mitochondrial fraction, and cytosol of all brain regions studied (Fig. 1A). CYP3A5 was detected in the microsomal fractions of all five regions (Fig. 1B). However, CYP3A5 was detected in the cytosolic fraction of only the cortex and hippocampus at significant levels and was absent from the mitochondrial fractions of any of the brain regions studied (Fig. 1B).

Immunoblots with the affinity-purified antibody raised against CYP3A4 revealed the presence of an additional band in the microsomal fractions of all five brain regions studied which migrated more slowly than CYP3A4 in liver microsomes and with an estimated molecular mass approximately 10 kDa higher than CYP3A4 (Fig. 1A). This band was not always observed, but did occur in the majority of CYP3A4 immunoblot experiments conducted on human brain microsomal samples.
A “spiking” experiment was conducted to determine whether the second band was another CYP3A isoform or whether the signal was due to differential migration of CYP3A4 protein as seen previously for CYP2D6 in brain microsomal samples (Mann et al., 2008). Addition of comparable amounts of liver microsomes to brain microsomal samples resulted in the detection of only a single band for CYP3A4 protein, indicating that the second band observed in the brain microsomal fractions of CYP3A4 immunobLOTS was due to altered protein migration (Fig. 2).

A lower molecular weight band was also observed in brain cytosol and microsomes probed with the antibody raised against CYP3A4 (Fig. 1A). N-terminal sequencing of the antigen affinity-purified from cortical microsomes revealed that the first eight amino acids of the putative degradation product were WGFYDGQQ, which corresponds with the sequence located between amino acids 72–79 of the human CYP3A4 protein. N-terminal sequencing of the principal CYP3A4 band revealed the starting sequence of human CYP3A4 (ALIP-DLAM). Notably, the corresponding sequences of CYP3A5 and CYP3A7 are WGTYEQL and WGIYDCQQ, respectively (amino acids 72–79), and DLIPNLAV for both enzymes (amino acids 2–9).

CYP3A4 and CYP3A5 Are Predominantly Expressed in the Somatic Region of Neuronal Cells. Immunohistochemical analysis using preimmune sera did not show any nonspecific neuronal staining (Fig. 3). CYP3A4 and CYP3A5 were expressed predominantly in the somatic region of neuronal cells, with expression frequently extending to the axonal hillock. Expression was also observed in neuronal axons (further down from the hillock and dendrites). The pattern of expression of the two forms differed within the same brain regions: CYP3A4 protein was detected from layer 3 through to layer 6 of the frontal cortex, whereas CYP3A5 expression was observed from layer 2 through to layer 5 (Fig. 3). CYP3A4 and CYP3A5 were both located in the hippocampus, where their expression appeared to be highest in the CA4 region (Fig. 4). Both P450s were expressed in the amygdala and basal ganglia (Fig. 5). By contrast, only CYP3A4 was detected in the cerebellum (Fig. 5) at significant levels, where immunoreactivity was localized in large pyramidal cells as well as in the small neuronal cells of the cerebellar cortex.

CYP3A4 Expression Is Elevated in Microsomal Fractions of Frontal Cortex from Alcoholic Subjects. CYP3A expression in brain samples from controls and alcoholic subjects was normalized to the α-tubulin signal, which was linear up to 30 μg (Supplemental Fig. 2). CYP3A4 expression was higher in the microsomal fractions of the frontal cortex of brains from individuals with a history of alcohol abuse than in age- and sex-matched controls (Fig. 6A) (P < 0.05; 95% confidence interval), but no difference was seen in CYP3A4 protein expression was localized in large pyramidal cells as well as in the small neuronal cells of the cerebellar cortex.

CYP3A4 Expression Is Elevated in Microsomal Fractions of Frontal Cortex from Alcoholic Subjects. CYP3A expression in brain samples from controls and alcoholic subjects was normalized to the α-tubulin signal, which was linear up to 30 μg (Supplemental Fig. 2). CYP3A4 expression was higher in the microsomal fractions of the frontal cortex of brains from individuals with a history of alcohol abuse than in age- and sex-matched controls (Fig. 6A) (P < 0.05; 95% confidence interval), but no difference was seen in CYP3A4 protein expression was localized in large pyramidal cells as well as in the small neuronal cells of the cerebellar cortex.

CYP3A4 Expression Is Elevated in Microsomal Fractions of Frontal Cortex from Alcoholic Subjects. CYP3A expression in brain samples from controls and alcoholic subjects was normalized to the α-tubulin signal, which was linear up to 30 μg (Supplemental Fig. 2). CYP3A4 expression was higher in the microsomal fractions of the frontal cortex of brains from individuals with a history of alcohol abuse than in age- and sex-matched controls (Fig. 6A) (P < 0.05; 95% confidence interval), but no difference was seen in CYP3A4 protein expression was localized in large pyramidal cells as well as in the small neuronal cells of the cerebellar cortex.

CYP3A4 Expression Is Elevated in Microsomal Fractions of Frontal Cortex from Alcoholic Subjects. CYP3A expression in brain samples from controls and alcoholic subjects was normalized to the α-tubulin signal, which was linear up to 30 μg (Supplemental Fig. 2). CYP3A4 expression was higher in the microsomal fractions of the frontal cortex of brains from individuals with a history of alcohol abuse than in age- and sex-matched controls (Fig. 6A) (P < 0.05; 95% confidence interval), but no difference was seen in CYP3A4 protein expression was localized in large pyramidal cells as well as in the small neuronal cells of the cerebellar cortex.

CYP3A4 Expression Is Elevated in Microsomal Fractions of Frontal Cortex from Alcoholic Subjects. CYP3A expression in brain samples from controls and alcoholic subjects was normalized to the α-tubulin signal, which was linear up to 30 μg (Supplemental Fig. 2). CYP3A4 expression was higher in the microsomal fractions of the frontal cortex of brains from individuals with a history of alcohol abuse than in age- and sex-matched controls (Fig. 6A) (P < 0.05; 95% confidence interval), but no difference was seen in CYP3A4 protein expression was localized in large pyramidal cells as well as in the small neuronal cells of the cerebellar cortex.
expression between brain samples from alcoholic subjects and controls in the mitochondrial fraction (unpublished data). No difference was observed in the expression of CYP3A5 in either the microsomal or mitochondrial fractions of the human frontal cortex between brains from alcoholic subjects and controls (representative data for microsomal fractions are presented in Fig. 6B).

Discussion

Despite their prominent role in drug metabolism, the study of CYP3A enzymes in the human brain has been hindered by the limited availability of brain tissue and of antibodies that can discriminate between CYP3A forms. Preliminary studies with form-specific short peptide antigens failed to generate antibodies of sufficient affinity (unpublished data). Therefore, in the current work, polyclonal antibodies raised to recombinant CYP3As were subject to affinity purification to obtain antibodies that showed markedly enhanced selectivity for CYP3A4 and CYP3A5. Immunoblots revealed expression of CYP3A4- and CYP3A5-related immunoreactivity to different extents in all five regions studied. The different patterns of expression seen with each antibody suggest preferential detection of two different antigens and the identity of the CYP3A4-immunoreactive band in the cortex was verified by N-terminal sequencing, supporting the contention that the antibodies are detecting the antigens against which they were raised. Limiting amounts of brain tissue prevented the use of this approach to confirm the identity of the anti-CYP3A4-reactive protein or anti-CYP3A5-reactive protein in other brain regions. However, the fact that only CYP3A4, CYP3A5, and CYP3A43 have been detected in brain at the mRNA level to date (Murray et al., 1995; McFadyen et al., 1998; Agarwal et al., 2008) also supports the contention that the antibodies used here are detecting CYP3A4 and CYP3A5. CYP3A7 expression in the liver has been

Fig. 3. Immunohistochemical detection of CYP3A4 and CYP3A5 expression in the human frontal cortex. CYP3A4 protein was detected in neurons of layers 3–6 of the frontal cortex. In contrast, CYP3A5 expression was observed in layers 2–5. The expression of CYP3A4 and CYP3A5 occurred predominantly in the somatic region of neuronal cells. Expression was also observed in neuronal axons and dendrites. (A and B) Negative controls (preimmune sera) for CYP3A4 and CYP3A5, respectively. (C, E, G, and I) Layers 3–6 treated with anti-CYP3A4 antibody. (D, F, H, and J) Layers 2–5 treated with anti-CYP3A5 antibody. Scale bar, 50 μm.

Fig. 4. Immunohistochemical detection of CYP3A4 and CYP3A5 expression in the human hippocampus. CYP3A4 and CYP3A5 were both located throughout the hippocampus; however, expression appeared to be highest in the CA4 region in both cases. (A, C, E, and G) CA1–CA4 treated with anti-CYP3A4 antibody. (B, C, F, and H) CA1–CA4 treated with anti-CYP3A5 antibody. (A’–H’) represent the same regions treated with the respective preimmune serum as a negative control. Scale bar, 50 μm.
mainly observed in fetal and perinatal samples, whereas it seems to decrease significantly in adults (Lacroix et al., 1997; Tateishi et al., 1997; Nishimura et al., 2003; Stevens et al., 2003). To our knowledge, this study provides the first evidence for the expression of CYP3A5 at the protein level in human brain, consistent with previous results at the transcriptional level (McFadyen et al., 1998).

CYP3A protein expression in subcellular fractions varied, with CYP3A4 immunoreactivity detected in both the mitochondrial and microsomal fractions. By contrast, CYP3A5 was absent from mitochondrial fractions but present in microsomes of all five regions tested, and in cytosolic fractions of the cortex and hippocampus. CYP3A expression has previously been observed in the mitochondrial fraction of whole rat brain extracts (Jayyosi et al., 1992). However, to our knowledge, human brain CYP3A expression has only previously been assessed and reported in microsomes (Pai et al., 2002). Although cross-contamination may have occurred between subcellular fractions during sample processing, distinct patterns of expression were seen in the two fractions arguing against the hypothesis that apparent P450 expression in mitochondrial and soluble fractions was due to contamination with microsomes. Importantly, it is now recognized that many “mitochondrial” P450s are also expressed in mitochondria, cytosol, and plasma membrane (Neve and Ingelman-Sundberg, 2008; Avadhani et al., 2011). Import into the mitochondrion involves an initial transit through the cytosol, which may explain the cytosolic localization of CYP3A4 in the present study, given the clear signal observed in the mitochondrial fraction. Moreover, brain P450 localization in soluble fractions might be ascribed to limited heme availability in this organ, which may impair enzyme insertion into the endoplasmic reticulum membrane (Meyer et al., 2002).

Three bands were clearly evident in frontal cortex microsomes incubated with anti-CYP3A4 antibody. The middle band was identified as full-length CYP3A4 by N-terminal sequencing, whereas the lower one appears to be a proteolysis product missing the first 71 residues (not prominent in the pooled sample used for Fig. 2) and may correspond to the functional truncated form found previously (Jeon et al., 2008). Variable migration of brain P450 proteins compared with the same forms in liver has been observed previously (Mann et al., 2008). We hypothesize that the upper band may represent protein subject to post-translational modification. Addition of comparable amounts of human liver microsomal protein to frontal cortex microsomes led to the loss of the upper band. We speculate that addition of liver microsomes led to restoration of the normal migration pattern due to the presence of an enzyme in liver microsomes that removed the post-translational modification. Further experiments are required to determine the basis to the slower migration of the upper band.

CYP3A isoforms were distributed differently between and within brain regions with anti-CYP3A4 immunoreactivity detected from layer 3 through to layer 6 of the frontal cortex, and anti-CYP3A5 immunoreactivity observed from layer 2 through to layer 5. Consistent with these observations, CYP3A4, CYP3A5, and CYP3A43 (but not CYP3A7) have been detected at the mRNA level in human cortex (Murray et al., 1995; McFadyen et al., 1998; Agarwal et al., 2008). CYP3A4 has also recently been detected at the mRNA and protein level in neurons and endothelial cells in temporal lobe from patients.
receiving anticonvulsants that are known inducers of hepatic CYP3A4 (Ghosh et al., 2010, 2011); however, no clear evidence for expression in microvessels was seen here or in a previous study (Dauchy et al., 2008).

The localization of both CYP3A4 and CYP3A5 in the pyramidal cell layer of hippocampal regions CA1–CA4 (Fig. 4) is in agreement with previous results at the transcriptional level. CYP3A-related mRNA was found in hippocampal regions CA1–CA3 (Pai et al., 2002), CYP3A43 mRNA was detected in the pyramidal neurons of the hippocampus (Agarwal et al., 2008), and CYP3A5 was detected but not quantified in hippocampus by real-time polymerase chain reaction (Dutheil et al., 2009). CYP3A4 and CYP3A5 were also both expressed in pyramidal neurons of the basal ganglia (Fig. 5) in accordance with expression of both forms at the transcriptional level in basal ganglia (McFadyen et al., 1998). Both CYP3A4– and CYP3A5-related immunoreactivity was seen in the amygdala by both immunoblotting and immunohistochemistry. To our knowledge, this is the first report of expression of any CYP3A form in human amygdala at either the protein or mRNA level. However, cyp3a11 protein has been detected in murine amygdala (Meyer et al., 2009).

CYP3A4 was detected immunohistochemically in the cerebellum at significant levels in the pyramidal and granular cell layers and in small neuronal cells of the cerebellar cortex. Agarwal et al. (2008) reported CYP3A4 but not CYP3A43 mRNA in pyramidal cells of the cerebellum, with CYP3A43 localized instead to granular cells and occasional cells in the molecular layer. Although CYP3A5 has not yet been detected at the mRNA level in cerebellum, trace signal was detected here with the antibody raised to CYP3A5; however, the thin structures with which it was associated could not be positively identified. This result is consistent with the weak band that was observed for CYP3A5 in cerebellum by immunoblotting, suggesting that the greater sensitivity of the Odyssey imaging system allows detection of the minimal expression of CYP3A5 in this tissue.

The shortage of human tissue and lengthy postmortem delays encountered with available brain samples obviated any investigation of whether the CYP3A forms detected here were functionally active. Regio-specific expression and differential induction of CYP3A in the brain may have implications for the site-specific disposition of xenobiotics and therefore influence the efficacy and/or toxicity of drugs (Britto and Wedlund, 1992; Pai et al., 2002). CYP3A4 but not CYP3A5 expression appeared to be upregulated in the frontal cortex of human alcoholic brain. Previous studies suggested that CYP3A expression is induced by ethanol in liver in vivo and ex vivo in primary hepatocytes (Hoshino and Kawasaki, 1995; Kostrubsky et al., 1995) and in the intestine of moderate alcohol consumers (Liangpun-sakul et al., 2005). However, to our knowledge, this is the first report of induction of CYP3A by alcohol in the human brain. It was beyond the scope of this study to confirm the mechanism underlying the observed induction of CYP3A4. However, one of the known mechanisms of ethanol-mediated P450 induction is enzyme stabilization, which has been observed both in liver and extrahepatically (Song et al., 1988; Koop and Tierney, 1990). Notably, this type of ethanol-mediated induction has also been observed with CYP3A4 expressed in HepG2 cells (Feierman et al., 2003). CYP3A4A metabolism of both endogenous and xenogenous compounds was inhibited by alcohol in human liver (Patki et al., 2004). Induction of CYP3A4 in the frontal cortex may represent a homeostatic response to alcohol inhibition of CYP3A4 activity, as alterations in CYP3A4 activity have been proposed to affect steroid-mediated signaling in the brain (Meyer and Gehrhaus, 2010). Changes in the dopaminergic system have also been linked to altered hepatic CYP3A4 expression in animals (Wójcikowski et al., 2007). Since chronic alcohol consumption can affect the dopaminergic system, it is conceivable that human CYP3A4 expression may be altered by the long-term effects of alcohol on neuronal signaling. Induction of CYP activity could affect localized drug or neurotransmitter metabolism with consequences for neural cell function. Further investigation is required to establish the mechanism underlying the changes in CYP3A4 expression in human brain and the possible biological significance of this effect.

In summary, this study has shown that CYP3A4 and CYP3A5 are expressed at the protein level in a number of human brain regions. The differential compartmentalization and response to chronic alcohol exposure seen here suggests that, assuming the proteins detected in the brain are active, CYP3A4 and CYP3A5 may have functionally different roles in the metabolism of endo- and xenobiotics.

Acknowledgments

The authors gratefully acknowledge the assistance of the New South Wales Tissue Resource Centre, which is supported by The University of Sydney, Neuroscience Institute of Schizophrenia and Allied Disorders, National Institute of Alcohol Abuse and Alcoholism, and New South Wales Department of Health, for the provision of the human brain samples, and the next of kin for providing informed written consent for the studies. Thanks are also extended to Dr. Rachel Tyndale for suggesting the experiment shown in Fig. 2, to Drs. Simon Worrall and M. Almira Correia for helpful comments on the manuscript, to Chris J. Wood for performing the N-terminal sequencing, and to Dr. Deanne Mitchell for assistance with fractionation of some of the antibodies.

Authorship Contributions

Participated in research design: Booth Depaz, Wilce, Gillam.

Conducted experiments: Booth Depaz, Toselli.

Contributed new reagents or analytic tools: Wilce.

Performed data analysis: Booth Depaz, Toselli, Gillam.

Wrote or contributed to the writing of the manuscript: Booth Depaz, Toselli, Gillam, Wilce.

References


