Recombinant Cytochrome P450 2D18 Metabolism of Dopamine and Arachidonic Acid

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ABSTRACT

The function of cytochrome P450 (P450) in the mammalian brain is not well understood. In an effort to further this understanding, this study identifies two endogenous substrates for P450 2D18. Previous reports have shown that this isoform is expressed in the rat brain, and the recombinant enzyme catalyzes the N-demethylation of the antidepressants imipramine and desipramine. By further examining the substrate profile of P450 2D18, inferences can be made as to potential endogenous P450 substrates. Herein we demonstrate the metabolism of the central nervous system-acting compounds chlorpromazine and chloroxazone with turnover numbers of 1.8 and 0.9 nmol/min/nmol, respectively. Because the four aforementioned P450s are expressed in the rat brain, and the recombinant enzyme catalyzes the N-demethylation of the antidepressants imipramine and desipramine. By further examining the substrate profile of P450 2D18, inferences can be made as to potential endogenous P450 substrates. Herein we demonstrate the metabolism of the central nervous system-acting compounds chlorpromazine and chloroxazone with turnover numbers of 1.8 and 0.9 nmol/min/nmol, respectively. Because the four aforementioned P450s present in nervous tissue is the first step to understanding their purpose in the brain as well as their potential use in the treatment of neurological disorders.

Parkinson’s disease is a neurodegenerative disorder of the basal ganglia that is characterized by loss of dopaminergic neurons within the substantia nigra pars compacta. Although it is unknown how this occurs, growing evidence suggests that much of the destruction of dopaminergic neurons is mediated by aberrant oxidation mechanisms. Increases in lipid hydroperoxides, hydrogen peroxide, and reactive catecholamine species have been implicated in neuronal destruction (Dexter et al., 1989; Spina and Cohen, 1989). Reactive catecholamine quinones and semiquinones are thought to lead to superoxide radicals, catecholamine-DNA and protein adducts, and imbalances in glutathione levels (Mattammal et al., 1995). Interestingly, there have been conflicting reports of P450 involvement in Parkinson’s disease (Coleman et al., 1996; Sabbagh et al., 1999).

Aminochrome is one culprit thought to be involved in dopaminergic neuron destruction. The oxidation of dopamine results in an electron-deficient o-quinone (Fig. 1, step 1) that is subject to nucleophilic attack by either its own side chain amino-group (step 2) or another nucleophile (step 4). Attack by the side chain amino-group results in the formation of leucochrome, which is then more easily oxidized to aminochrome (step 3) (Tse et al., 1976). Studies on aminochrome have shown that prostaglandin H synthetase (PGS) can co-oxidize dopamine to aminochrome during the conversion of AA to prostaglandin H₂ (Hastings, 1995) and that PGS can co-oxidize dopamine in the presence of peroxide without AA (Mattammal et al., 1995). Recently, it has been shown that P450 1A1 can oxidize dopamine to aminochrome in a perox-
ide-dependent manner (Segura-Aguilar, 1996). More recently, one group has shown that human P450 2D6 can hydroxylate the trace amine, tyramine, to dopamine in a reductase-dependent manner (Hiroi et al., 1998).

In this report, we provide evidence that dopamine is a substrate for the recently cloned and purified recombinant P450 2D18, an isofrom of P450 that is expressed, in vivo, in brain and kidney tissue but not in liver tissue (Kawashima and Strobel, 1995; Thompson et al., 1998). Indirect evidence for this activity is provided by examination of the substrate profile for P450 2D18. This isofrom was previously shown to metabolize imipramine and desipramine (Thompson et al., 1998), whereas herein we report the metabolism of chlorpromazine (CPZ) and chlorzoxazone. All four of the aforementioned pharmaceuticals have a mechanism of action involving binding to neurotransmitter receptors. Furthermore, it is speculated that structural similarities between CPZ and dopamine mediate the binding of the former to D2 dopamine receptors. Similar structural comparisons can also be made for imipramine, desipramine, and chlorzoxazone. In this study, direct evidence for P450 2D18-specific metabolism of dopamine is provided by binding and catalysis data.

In addition to the examination of dopamine metabolism, we provide evidence for 2D18 hydroxylation and epoxygenation of the lipid-derived mediator AA. P450s have been shown to metabolize AA to several metabolites, including 5,6-, 8,9-, 11,12-, and 14,15-epoxyeicosatrienoic acid (EET) and 16-, 17-, 18-, 19-, and 20-hydroxyeicosatetraenoic acid (Capdevila et al., 1981; Fitzpatrick and Murphy, 1989; Capdevila et al., 1995). Many of these metabolites are thought to have physiologically significant roles including hormone regulation and renal Na+/K+ -ATPase activity (Kutsya et al., 1983; Snyder et al., 1983; Negro-Vilar et al., 1985; Ominato et al., 1996). Several studies have shown that the epoxygenase pathway metabolites (EETs) increase cerebral blood flow in rabbits and cats and enhance cerebral microvascular smooth muscle Ca2+ -activated K+ channels in cultured rat brain astrocytes (Ellis et al., 1990; Amruthesh et al., 1992).

Because of the recent reports showing P450 activity toward catecholamines and recent reports of AA metabolites affecting membrane and receptor structure, we chose to demonstrate these reactions together because of their potential relevance to neurophysiology. Furthermore, we provide evidence for an interaction between these two substrates that might be of significance under aberrant physiological conditions such as Parkinson’s disease.

Materials and Methods

Chemicals. All chemicals were from Sigma Chemical Co. (St. Louis, MO) except the following: dopamine was purchased from Research Biochemicals International (Natick, MA), AA hydroperoxides were obtained from Cayman Chemical (Ann Arbor, MI), 6-hydroxychloroxazone was acquired from Ultrafine (Manchester, England), and [3H]dopamine was purchased from New England Nuclear (Boston, MA).

Enzyme Assays. Recombinant P450 2D18 and 4F5 were purified as previously reported (Kawashima et al., 1997; Thompson et al., 1998). CPZ and chlorzoxazone assays were carried out as previously described by Boehme and Strobel (1998) and Lucas et al. (1996), respectively. Turnover numbers were determined by subjecting known amounts of authentic metabolites to the extraction and HPLC procedures used for the enzymatic reactions. AA metabolism and stereochemistry were performed as described previously (Capdevila et al., 1991). Aminochrome reactions were carried out in 1.0-ml reaction mixtures containing 0.1 M Tris-acetate, pH 7.4, 0.1 mM EDTA, and 20% glycerol. Except where noted, reactions containing 300 μM dopamine and 200 pmol of P450 2D18 were preincubated at 37°C for 5 min, and reactions were initiated with the addition of 40 mM t-butyl hydroperoxide (t-BOOH). Aminochrome formation was measured in a quartz cuvette by following the absorbance at 475 nm in a Hewlett Packard 8452A diode array spectrophotometer at 37°C for 30 min and quantified using extinction coefficients of ε = 3058 M⁻¹ cm⁻¹ for aminochrome formation and ε = 14,350 M⁻¹ cm⁻¹ for 6-hydroxychlorzoxazone (Segura-Aguilar and Lind, 1989). AA hydroperoxide-mediated reactions were carried out with a 1:3:1 ratio of 5(S), 12(S), and 15(S)-hydroperoxyeicosatrienoic acid (72 nM final concentration). Reductase-supported reactions were carried out using a 1:1 M ratio of P450 and P450-reductase, L-α-dilauroyl phosphatidylcholine, and 1.0 mM NADPH. Reversed phase (RP) HPLC reactions were carried out with the same concentrations of substrate and t-BOOH, except only 100 pmol of P450 2D18 was added to reaction mixtures. After 5 min of preincubation at 37°C, reactions were initiated with 40 mM t-BOOH. Samples (50 μl) were injected onto a 5-μm, 4.6 × 250-mm TSK gel ODS-120T column (TOSOHAS, Montgomeryville, PA) without extraction or modification. The mobile phase consisted of 0.1 M KH2PO4, pH 7.0, for 10 min, followed by a 10-min linear increase of KH2PO4/methanol (50:50, v/v) to 40%. Samples were run at a flow rate of 1.0 ml/min for 30 min. The same conditions were used in photodiode array analysis except that 100 μl of sample was injected onto the column. Each injection, the baseline at 225 nm was stabilized with 0.15M KH2PO4, pH 3.0. Control, heme/Fe3+ -mediated aminochrome reactions were measured at dual wavelengths of 225 and 475 nm for dopamine and aminochrome, respectively. Due to a decrease in sensitivity at the visible wavelength from UV interference...
ence, enzymatic samples were measured only at 475 nm to increase the sensitivity for aminochrome detection.

**Determination of Dopamine Conjugation to BSA.** Reactions were carried out in 0.5-ml volume containing 50 pmol of P450 2D18, 600 μM dopamine, 10 μCi of [3H]dopamine, 1 mg/ml BSA, 40 mM t-BOOH, and 1 mM glutathione (GSH) where applicable. Reactions were incubated for 30 min at 37°C, precipitated with 71 μl of 70% trichloroacetic acid, and centrifuged (20 min, 14,000g, 4°C). The pellet was resuspended in 0.2 ml of 0.1 M NaOH. The reactions were then spotted onto glass microfiber filters in a vacuum manifold. In brief, filters were prewetted with 4 ml of 10% trichloroacetic acid (TCA), and 0.1 ml of sample was applied; washed with 4 ml of 10% TCA, 12 ml of 2.5% TCA in 50% methanol, and 4 ml of methanol; dried; and dissolved overnight in scintillation fluid. The remaining sample was used to determine protein concentration. Statistical significance was determined using the Student's t test.

**Binding Assays and Analysis of Protein Stability.** Heme destruction was measured by following the absorbance spectrum of protein in the presence or absence of 40 mM t-BOOH. Binding assays were performed by dividing 1 nmol of P450 2D18 in phosphate buffer into two cuvettes. Substrate was then titrated into the sample cuvette, and an equal volume of methanol was titrated into the reference cuvette. ΔA values were calculated by double-reciprocal plots of concentration versus ΔA

### Results

**Metabolism of CPZ and Chlorzoxazone.** We previously reported that P450 2D18 can metabolize the dopamine re-uptake inhibitor antidepressants imipramine and desipramine (Thompson et al., 1998). Although we propose that in situ brain-mediated antidepressant metabolism may have some role in the alleviation of depression, it also suggests that P450 2D18 may metabolize other central nervous system (CNS)-acting compounds. We therefore investigated whether P450 2D18 could metabolize the neuroleptics CPZ and haloperidol. Given the structural similarities between imipramine and CPZ, we hypothesized that P450 2D18 would preferentially catalyze the demethylation of CPZ as opposed to hydroxylation. Indeed, Fig. 2 (A and B) show that P450 2D18 catalyzes the N-demethylation of CPZ to nor-CPZ with an apparent turnover of about 1.8 nmol/min/nmol. Interestingly, P450 2D18 exhibited no activity toward haloperidol (data not shown).

Previous studies have shown that human P450 2D6 can hydroxylate the monoamine tyramine to the catecholamine neurotransmitter dopamine (Hiroi et al., 1998). We have previously reported that the dopamine reuptake inhibitor GBR-12935 can inhibit P450 2D18 N-demethylation of imipramine (Thompson et al., 1998), and we have also observed that dopamine can inhibit other P450 2D18 activities such as the N-demethylation of CPZ (data not shown). Although previous reports have used sensitive HPLC-electrochemical detection methods, we were unable to detect tyramine hydroxylation by P450 2D18 using less sensitive HPLC-UV detection. Of the P450 2D18 activities we have observed to date, we have found little or no hydroxylated metabolite formation despite the potential for such activities to occur. For this reason, we chose a sensitive RP-HPLC assay to test whether chlorzoxazone could be hydroxylated by P450 2D18. Chlorzoxazone is a muscle relaxant that acts within the spinal cord to block polysynaptic transmission, and it has been used as a model substrate for P450 2E1 (Lucas et al., 1996). Figure 2C shows the RP-HPLC chromatograms indicating an apparent turnover number of about 0.9 nmol/min/nmol for chlorzoxazone by P450 2D18. These data show that P450 2D18 can catalyze dopamine receptor-binding compounds such as CPZ and catalyze the hydroxylation of smaller neurotransmitter-like compounds such as chlorzoxazone.

**Peroxide-Supported Oxidation of Dopamine.** In 1996, Segura-Aguilar (1996) suggested that liver P450 1A2 could oxidize dopamine in a peroxide-dependent manner. Because we have previously observed dopamine inhibition of other enzymatic reactions and we have shown that chlorzoxazone is a substrate for P450 2D18, we tested the hypothesis that peroxides could support dopamine oxidation by P450 2D18. In accordance with previously published data, we tested the effect of 40 mM t-BOOH for its ability to support P450-mediated oxidation of dopamine by following aminochrome absorbance at 475 nm over time (Segura-Aguilar, 1996). Figure 3A shows the effect of native and heat-denatured P450 2D18 on the formation of aminochrome in the presence of 40 mM t-BOOH. These data suggest that dopamine oxidation to aminochrome is both P450- and peroxide-dependent. Aminochrome formation was not detected in control reactions lacking individual components, reactions containing only 4 mM t-BOOH, or in reactions substituting P450-reductase and NADPH for peroxide (data not shown).

In 1997, Pezzella et al. (1997) showed that peroxides could oxidize dopamine to aminochrome and 6-hydroxydopamine quinone in a Fe2+-dependent manner. However, in their experiments, they used 350 μM Fe2+. In our reactions, the only possible source of iron would be from destruction of the P450 enzyme. The maximal possible concentration of heme would then be 0.2 μM in our system. To rule out the possibility that aminochrome formation was due to heme destruction by t-BOOH, we measured the protein-heme complex stability in the presence of 40 mM t-BOOH for more than 25 min (Fig. 3C). Although there is a decrease in absorption at 418 nm suggesting some heme destruction, the protein remains relatively stable in the presence of t-BOOH. These data suggest that the majority of heme remains bound to the protein; thus, Fe2+-mediated oxidation of substrates by heme "leakage" is unlikely. Furthermore, our reaction buffer contained 0.5 mM EDTA that serves as an iron chelator. Interestingly, 40 mM t-BOOH had a more marked effect on heme stability than purified P450 1A1. Much of the P450 1A1 heme was destroyed immediately, whereas only about 20% remained intact for more than 25 min (data not shown). On the other hand, purified P450 4F5, like P450 2D18, was quite stable in 40 mM t-BOOH (data not shown), suggesting an isozyme dependence on either the sensitivity to or affinity for t-BOOH.

Further evidence that dopamine oxidation is not due to Fe2+ exudation from the enzyme is shown in Fig. 3B. As mentioned previously, P450 1A1 was more sensitive to t-BOOH than P450 2D18, whereas P450 4F5 was also stable in the presence of 40 mM t-BOOH. Figure 3B shows that relative to P450 1A1 and 4F5, P450 2D18 forms aminochrome more readily. The formation of aminochrome by P450 1A1 is in agreement with previous work showing aminochrome formation by P450 1A2 (Segura-Aguilar, 1996). The fact that P450 4F5 does not support aminochrome formation despite its stability in 40 mM t-BOOH lends further evidence for aminochrome formation being enzymatically catalyzed and for enzyme specificity.
Data for the initial 5 min of the reactions are not shown due to interference from the spectral interactions of P450 with t-BOOH. It is well known that binding of a substrate to the P450 active site leads to changes in the heme spin state that can be followed spectrally by measuring the $\Delta A_{380-420}$; this value increases with increasing substrate concentration. The absorbance between 450 and 500 nm can also undergo changes. When we tested the effect of 40 mM t-BOOH on P450 4F5, which does not support aminochrome formation, we observed an immediate increase in absorbance at 475 nm that was followed by a 5-min recovery to baseline (data not shown). The fact that reactions containing native P450 2D18 first show a decrease in absorbance, followed by a steady increase in $A_{475}$ around 5 min after the addition of t-BOOH, is consistent with aminochrome formation. Furthermore, this effect was nearly abolished in reactions containing no enzyme and in reactions containing enzyme with only 4 mM t-BOOH. Likewise, P450 1A1 does not show the aforementioned effect during the initial 5 min because most of the enzyme is destroyed immediately on the addition of t-BOOH. Therefore, under these assay conditions, the binding of t-BOOH precludes us from detecting aminochrome during the initial 5 min of the reaction.

**RP-HPLC for Aminochrome Detection.** Previous studies have shown that dopamine can be oxidized to aminochrome in the presence of $\text{Mn}^{3+}$-pyrophosphate or in the presence of iron and peroxides (Segura-Aguilar and Lind, 1989; Pezzella et al., 1997). To prepare aminochrome, we used a mixture containing 300 $\mu$M dopamine, 400 $\mu$M hemichloride, and 40 mM t-BOOH in Tris buffer that resulted in

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**Fig. 2.** RP-HPLC chromatograms of CPZ and chlorzoxazone metabolism with accompanying structures. CPZ metabolism after 20 min in the absence (A) and presence (B) of 0.5 mM NADPH. The peaks with retention times of 5 and 10 min correspond to authentic CPZ and nor-CPZ, respectively. C, chlorzoxazone metabolism after 25 min in the absence of NADPH (solid line) and in the presence of 0.5 mM NADPH (dashed line). The peak with retention time 6.5 min corresponds to authentic 6-hydroxychlorzoxazone.
the appearance of a pink chromophore that had two absorbance maxima at 300 and 475 nm. When subjected to RP-HPLC, a peak corresponding to authentic dopamine and an unknown peak with retention time 18 min were observed. Two chromatograms of the same HPLC run are shown in Fig. 4. Although dopamine is the major peak observed at 225 nm, the peak with retention time 18 min is more readily observed at 475 nm, an $\lambda_{\text{max}}$ characteristic for aminochrome (Fig. 4A). Control mixtures lacking either hemichloride or $t$-BOOH did not produce the pink chromophore or the peak with retention time 18 min (Fig. 4, B and C). These data are consistent with the notion that the peak with retention time 18 min is aminochrome. As mentioned earlier, absorbance at 475 nm in this reaction is indicative of aminochrome. Spectral extraction of the putative aminochrome peak using an HPLC-photodiode array (PDA) spectrophotometer is shown in the inset of Fig. 5A, which shows two absorbance maxima at 300 and 475 nm. This is in agreement with previously published spectra for aminochrome observed during Mn$^{3+}$-pyrophosphate oxidation of dopamine, and during the co-oxidation of dopamine in AA-mediated prostaglandin E$_2$ synthesis (Segura-Aguilar and Lind, 1989; Mattammal et al., 1995). These data suggest that aminochrome formation can be followed using this chromatography strategy.

To increase the sensitivity of aminochrome detection, enzymatic reactions were monitored at a single wavelength, 475 nm, by RP-HPLC with the exception of the series of experiments using the HPLC-PDA system. We chose to monitor at 475 nm because it biases for the detection of aminochrome without the interference of peaks detected at lower wavelengths. Figure 5, A and B, shows a representative set of chromatograms for aminochrome formation in the presence of 100 pmol of native or heat-denatured P450 2D18. We found that this method was quite sensitive in that the activity could be measured by applying 50 $\mu$l of the reaction mixture directly to the column without extraction. Taken together, these data show that P450 2D18 can oxidize dopamine to the quinone, aminochrome, in a peroxide-dependent manner.

**Effect of GSH on P450 2D18 Oxidation of Dopamine.**

Previous studies have suggested that dopamine quinones can
bind to cysteinyl residues in proteins. To assess further the consequences of dopamine oxidation by P450 2D18, we chose to investigate whether protein-bound dopamine was increased in the presence of P450 2D18. Figure 6A shows the effect of GSH on aminochrome formation by P450 2D18. A similar effect is shown in Fig. 6B, which shows the incorporation of radiolabeled dopamine into BSA. The graph indicates that there is a certain level of background or chemically mediated quinone formation and further indicates that there is an increase \( (P = .07) \) in radiolabeled protein consonant with the hypothesis that P450 2D18 can generate dopamine \( \alpha \)-quinone. The graph also shows a significant \( (*P < .05) \) decrease in radiolabeled protein in the presence of 1 mM GSH and P450 compared with P450 alone, which is consistent with the notion that GSH binds to dopamine \( \alpha \)-quinone before cyclization can occur. The presence of radiolabeled

**Fig. 4.** RP-HPLC chromatograms of chemically oxidized dopamine measured at dual wavelengths. Top and bottom traces correspond to 225 and 475 nm, respectively. A, reaction mixture contains 300 \( \mu \)M dopamine, 0.4 \( \mu \)M hemichloride, and 40 mM \( t \)-BOOH. B, reaction mixture without hemichloride. C, reaction mixture without \( t \)-BOOH. Spectral extraction of the peak with retention time 18 min (see inset of Fig. 5) corresponds to previously published spectra for aminochrome.
protein even in the absence of P450 2D18 is not surprising in
that it is known that dopamine will oxidize spontaneously
even at room temperature.

Formation of Aminochrome by P450 2D18 and Lipid Peroxides. Because we have observed that P450 2D18 can
catalyze both the ω-hydroxylation and epoxygenation of AA
(see later), we chose to investigate whether AA hydroperox-
ides could serve as cofactors to support the P450 2D18-me-
diated oxidation of dopamine. To examine this activity, we
first performed binding assays for AA and dopamine to find
optimal substrate and cofactor concentrations for this reac-
tion. Figure 7A is a double reciprocal plot yielding apparent
$K_S$ values of 142 and 678 μM for AA and dopamine, respec-
tively. In accordance with these $K_S$ values, we chose to use 1
mM dopamine and 140 μM AA hydroperoxides in our reac-
tion mixtures. As reported by Wang and Liehr (1994), how-
ever, we observed activity only when AA hydroperoxide was
present in nanomolar concentration. This may indicate that
the $K_S$ values for lipid peroxides are lower than their parent
forms. Figure 7B shows the results of aminochrome forma-
tion in the presence of 72 nM AA. Although native and
denatured reactions appear to have similar slopes up to
about 20 min, the erratic fluctuations and the decreased
absorbance after 20 min with denatured enzyme suggest that
this absorbance is artifact. This notion is further supported
by Fig. 7, C and D, which shows plots of residuals versus
predicted absorbance values based on linear regression of the
absorbance (λ = 475 nm) recorded from 5 to 15 min shown in
Fig. 7B. These two figures indicate that the absorbance in the
presence of denatured enzyme is inconsistent with a linear
increase in aminochrome formation. The coefficients of de-
determination, $r^2$, for the denatured and native reactions (from
5 to 15 min) are 0.871 and 0.991, respectively. Furthermore,
$r^2 = 0.329$ and 0.984 over the entire course of the reactions
when using denatured and native enzyme, respectively ($n = 2$).
These data suggest that the denatured reactions do not fit
a linear regression model well, further indicating that the
absorbance is artifact. In contrast, the data for the native
reactions are consistent with a linear regression model and
an enzymatic reaction.

Catalytic Activity toward AA. The vasoactive properties
described for the EETs in brain microcirculation (Amruthesh
et al., 1992; Harder et al., 1998), the effect of these metabo-
lites on the ion channel activities of nerve cells, and the
established role of the CYP 2D gene family isoforms in AA
metabolism (Oliw, 1994; Capdevila et al., 1995), prompted us
to 1) characterize the metabolism of AA by the purified and
reconstituted P450 2D18 and 2) determine the in vivo forma-
tion of the EETs in whole brain tissue. As described in the
radiochromatograms in Fig. 8, P450 2D18 catalyzes the
NADPH-dependent oxidation of AA (apparent turnover = 0.32 nmol/min/nmol) to metabolites with the HPLC retention times of synthetic 8,9-, 11,12-, and 14,15-EET and 15- and 19-hydroxyeicosatetraenoic acids. By comparison, P450s 2C29, 2C37, 2C38, 2C39, and 2C40 have turnover numbers of 0.34, 1.1, 5.2, 1.1, and 0.15 nmol/min/nmol, respectively (Luo et al., 1998). Table 1 summarizes the isomeric and enantiomeric metabolites of P450 2D18-mediated oxidation of AA. These product profiles demonstrate the ability of P450 2D18 to function both as an active AA epoxygenase and \( \omega \)-1 hydroxylase (Capdevila et al., 1995). Similar product profiles have been previously reported for rat P450s 2C11 and 2E1 (Oliw, 1994; Capdevila et al., 1995; Makita et al., 1996).

To assess the potential functional relevance of this P450 2D18 metabolic activity, brain lipid extracts were isolated and assayed for the in vivo presence of EETs in tissue analyzed by a combination of stable isotope dilution and gas chromatography-mass spectrometry techniques (Karara et al., 1989). As shown in Table 2, the most abundant regioisomer in rat brain was the 8,9-EET. The brain concentration of total EETs is significantly higher than that reported for rat liver and hypothalamus (Karara et al., 1989). Although the brain EET regioisomeric composition is different than that of the EETs generated by P450 2D18, it has been demonstrated that organ EET levels are determined by, among other things, rates of P450-dependent biosynthesis, hydration and excretion as dihydroxyeicosatrienoic acids, and esterification to endogenous glycerophospholipid pools (Karara et al., 1991).

### Discussion

This report further characterizes the metabolism of CNS-acting compounds by P450 2D18. Herein we have shown that P450 2D18 catalyzes the N-demethylation of CPZ to nor-CPZ with a turnover number of about 1.8 nmol/min/nmol, which is nearly 10 times the rate reported (Boehme and Strobel, 1998) for purified P450 1A1 and 2B1 (0.21 and 0.26 nmol/min/nmol, respectively). Interestingly, P450 2B1 also catalyzed the formation of CPZ-sulfoxide as well as another unidentified CPZ metabolite, yet nor-CPZ was the only product formed by P450 2D18. This is somewhat surprising, given that P450 2D18 shares more sequence identity with P450 2B1 than 1A1. Also, P450 2D18 showed no activity toward haloperidol (data not shown) despite the fact that both P450 1A1 and 2B1 catalyze the reduction and dealkylation of haloperidol to form reduced haloperidol and 4-(4-chlorophenyl)-4-hydroxypiperidine, respectively (Boehme and Strobel, 1998).

In 1998, it was reported that human P450 2D6 can hydroxylate monoamines to catecholamines. Although we were unable to detect such activity with P450 2D18, we were able to detect the hydroxylation of the CNS-acting compound chlorzoxazone with a turnover number of about 0.9 nmol/min/nmol. This activity, along with reports of P450-mediated oxidation of dopamine set the stage for examining P450 2D18-mediated oxidation of dopamine to aminochrome.

These data show that P450 2D18 and, to a lesser extent, other P450s are capable of oxidizing dopamine in a peroxide-dependent manner. It is difficult to accurately quantify aminochrome formation for the reasons mentioned under Results. Despite these limitations, we estimate the apparent turnover of dopamine to be about 8.2 nmol/min/nmol. This turnover number is 130 times greater than what we observed in reactions containing up to 40 mM \( \text{H}_2\text{O}_2 \), suggesting that aminochrome formation was not driven by \( t\text{-BOOH} \)-generated \( \text{H}_2\text{O}_2 \). Recently, it has been shown that human P450 2D6 can metabolize tyramine to dopamine in an NADPH-dependent manner (Hiroi et al., 1998). Our data raise the possibility that perhaps dopamine oxidation by P450 2D6 could have greater consequence within the brain because tyramine is a trace amine, whereas dopamine concentration within dopaminergic cells is reported to be in millimolar concentration. Perhaps peroxide-mediated oxidation of dopamine by a host of cellular proteins like PGS and P450 is a contributing factor to neuronal degeneration. This is a likely

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**Fig. 6.** Effect of glutathione on oxidation of dopamine by P450 2D18. A, measurement of \( A_{475} \) in the presence (solid line) and absence (dashed line) of 0.2 mM GSH. These reactions were carried out with 100 pmol of enzyme and 1 mM dopamine. B, measurement of the amount of [3H]dopamine bound to BSA. Reactions containing 50 pmol of enzyme contained more radioactivity than reactions without enzyme \( (P < .07, df = 4) \) or reactions containing enzyme and 1 mM GSH \( (*P < .05, df = 3) \).
possibility given that CYP 2D mRNA and protein reactivity have been mapped to the substantia nigra, where dopaminergic neuronal degeneration is known to occur in Parkinson’s disease (Riedl et al., 1999).

Peroxide-supported oxidation of dopamine is not unlikely because it has been shown that oxidative stress increases during dopaminergic neuron degeneration. Furthermore, fatty acid hydroperoxides are also increased during or as a result of dopaminergic cell death. Interestingly, fatty acid hydroperoxides seem to support P450 reactions at very low (nanomolar) concentrations. We estimate the turnover of dopamine to be about 1.0 nmol/min/nmol in the presence of 72 nM AA hydroperoxide. This is in close agreement with fatty acid hydroperoxide concentrations in other P450-mediated oxidation reactions (Wang and Liehr, 1994).

In the presence of native enzyme, no aminochrome formation was detected in the presence of glutathione. Studies have shown that both glutathione and cysteine residues can bind to dopamine o-quinone at the sixth position to inhibit cyclization to aminochrome (Tse et al., 1976). Such additions occur three orders of magnitude faster than the cyclization of dopamine o-quinone. Similarly, other studies involving PGS have shown increases in dopamine-protein adducts during the metabolism of AA in the presence of dopamine (Hastings and Zigmond, 1994; Hastings, 1995; Mattammal et al., 1995). We have provided evidence that [3H]dopamine binding to BSA is increased in the presence of P450 2D18 and that this binding is significantly reduced in the presence of GSH. Although P450 activities have not been shown to trigger neuronal degeneration, it is possible that PGS and P450 oxidations could sway the balance between oxidative stress and antioxidant protection at intermediate or late stages of neuronal cell degeneration. It is important to realize that ascorbic acid can re-reduce dopamine o-quinone back to dopamine, but as Tse et al. (1976) have pointed out, even equimolar amounts of ascorbic acid cannot completely inhibit nucleophilic additions to dopamine o-quinone in vitro. With the wide array of available nucleophiles in vivo, it is likely that dopamine adducts of lipid, DNA, protein, and GSH could severely impair cell physiology of dopaminergic neurons.

The NADPH-dependent metabolism of AA by P450 to regioisomeric epoxyeicosatrienoic acids and ω/ω-1 alcohols is
well established (Schwartzman et al., 1996; Rahman et al., 1997; Harder et al., 1998). Interest in these reactions has been stimulated by 1) the demonstration of in vivo EET formation and 2) the wide spectrum of powerful biological activities associated with these compounds. The presence of endogenous EET pools in the rat hypothalamus has been demonstrated, and EET roles in dopamine signaling and peptide hormone release from median eminence nerve terminals have been suggested.

In contrast to other P450s, the CYP 2 family seems to be highly enantioselective in epoxidation reactions. As shown in Table 1, the more predominately formed 11,12- and 14,15-EET are also selectively oxidized in about the same ratio. Examination of AA metabolism by other P450s expressed in brain can further lend support to the significance of P450 2D18 metabolism of AA. Interestingly, we observed very little metabolism of AA by our purified recombinant P450s 3A9, 4F4, and 4F5 (data not shown). Purified P450 1A1, on the other hand, primarily catalyzes the hydroxylation of AA (data not shown). Studies have shown that regiochemical and stereochemical selectivity can be experimentally altered, in vivo, by drug treatment. That is to say that P450 inducers such as phenobarbital can alter the AA metabolite profile. Interestingly, we found no significant change in the rat brain EET regiochemistry and stereochemistry after phenobarbital treatment. This is more interesting in light of the fact that CYP 2D18 is not inducible by phenobarbital. These data suggest that P450 2D18 may be responsible for some portion of the 11,12- and 14,15-EET observed in the rat brain (Table 2). In summary, these data characterize brain P450 2D18 as an active AA epoxygenase and suggest a role for this enzyme in brain EET biosynthesis and AA bioactivation.

The ability of P450 2D18 to bind and metabolize AA has

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**Table 1**

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Retention Time</th>
<th>Percentage of Total</th>
<th>Enantiomers (R:S; S,R)</th>
</tr>
</thead>
<tbody>
<tr>
<td>19-OH</td>
<td>14</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>15-HETE</td>
<td>19</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>8,9-EET</td>
<td>24</td>
<td>7</td>
<td>48; 52</td>
</tr>
<tr>
<td>11,12-EET</td>
<td>23</td>
<td>40</td>
<td>76; 24</td>
</tr>
<tr>
<td>14,15-EET</td>
<td>22</td>
<td>21</td>
<td>71; 29</td>
</tr>
</tbody>
</table>

19-OH, 19-hydroxyeicosatetraenoic acid; 15-HETE, 15-hydroxyeicosatetraenoic acid.

**Table 2**

<table>
<thead>
<tr>
<th>Regioisomer</th>
<th>Retention Time</th>
<th>Percentage of Total</th>
<th>Enantiomers (R:S; S,R)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8,9-EET</td>
<td>24</td>
<td>38</td>
<td>47; 53</td>
</tr>
<tr>
<td>11,12-EET</td>
<td>23</td>
<td>29</td>
<td>46; 54</td>
</tr>
<tr>
<td>14,15-EET</td>
<td>22</td>
<td>33</td>
<td>53; 47</td>
</tr>
</tbody>
</table>

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Fig. 8. Metabolism of AA by P450 2D18 in the absence of NADPH (AA) and in the presence of 1 mM NADPH (B). Peaks at 14, 19, 22, 23, and 24 min correspond to authentic metabolites. See Table 1 for metabolite identifications.


Oliv E (1994) Oxidation of polyunsaturated fatty acids by Cytochrome P450 2D18 can metabolize both dopamine and AA. Further investigations into these activities by the CYP 2D family as well as other families are highly warranted.

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References