CYTOCHROME P-450 AND NADPH CYTOCHROME C REDUCTASE IN RAT BRAIN: FORMATION OF CATECHOLS AND REACTIVE CATECHOL METABOLITES

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SUMMARY: Microsomes isolated from whole rat brain were found to contain cytochrome P-450 (0.025 to 0.051 nmoles/mg) and NADPH cytochrome c reductase activity (26.0 to 55.0 nmoles/mg/min). The oxidation of estradiol to a reactive metabolite that became covalently bound to rat brain microsomal protein was inhibited 63% by an atmosphere of $CO:O_2$ (9:1), indicating the involvement of a cytochrome P-450 oxygenase. In contrast, this atmosphere had no effect on the binding of either the catechol estrogen, 2-hydroxy-estradiol, or several catecholamines to rat brain microsomes. An antibody prepared against NADPH cytochrome c reductase was found to decrease significantly both the formation of 2-hydroxyestradiol from estradiol by rat brain microsomes and the covalent binding of the catechol estrogen and catecholamines to rat brain microsomal protein.

INTRODUCTION

Although several studies have suggested that brain contains enzymes which metabolize drugs by reactions that are known to be catalyzed by liver microsomal cytochrome P-450 enzymes, the analytical methodology required to demonstrate these slow reactions has been inadequate until recently. It now appears that rat brain can N-demethylate morphine (1), convert parathion to paraoxon (2) and hydroxylate estrogens to 2-hydroxyestrogens (3). Although the finding that carbon monoxide inhibits the formation of paraoxon (2) and 2-hydroxyestrogens (3) suggests that cytochrome P-450 may catalyze these reactions, there has been no direct proof for this view. Indeed, attempts to detect cytochrome P-450 have failed (2).

The present study shows that the presence of NADPH cytochrome c reductase and cytochrome P-450 in brain. It also presents evidence that these enzymes catalyze the conversion of estradiol to 2-hydroxyestradiol

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Table I Comparison of the levels of cytochrome P-450 and NADPH cytochrome c reductase activity in rat brain and liver

Results are the mean ± S.E of 6 experiments, each experiment involving organs pooled from at least 20 male rats

Tissue	Cytochrome P-450[a]	NADPH Cytochrome c Reductase[b]
Brain	0.036 ± 0.04	38.9 ± 4.1
Liver	0.98 ± 0.06	121.0 ± 9.0

a. nmoles/mg microsomal protein
b. nmoles of cytochrome c reduced/mg microsomal protein/min.

and that superoxide converts the 2-hydroxyestradiol and other catechols to chemically reactive metabolites.

MATERIALS AND METHODS

All radiolabeled chemicals used in this study were obtained from companies listed previously (4,5). Antibody against NADPH cytochrome c reductase was prepared as previously described (6).

Whole rat brains were dissected from male Sprague-Dawley rats (Taconic Farms 180-220g) and homogenized with 3 volumes of nitrogen-deaerated 1.15 M KCl-0.02 M Tris buffer containing 0.5 mM dithiothreitol, 0.2mM EDTA and 15% by volume of glycerol. The homogenate was centrifuged at 19,000 x g for 20 min in a Serval RC-2 centrifuge. Microsomes were isolated and resuspended as described previously (5). The cytochrome P-450 was measured by the method of Omura and Sato (7). The CO-reduced difference spectra were obtained on Aminco DW-2 Spectrophotometer by injecting NADPH (10 µl, 0.3 mM) into one of two CO-gassed anaerobic cuvettes. Anaerobiosis was insured by glucose/glucose oxidase system as described by Matsubara et al (8). Difference spectra were also obtained with rat brain and liver microsomes and various concentrations of 17β-estradiol. NADPH cytochrome c reductase activity was monitored according to the method of Phillips and Langdon (9). Covalent binding experiments using rat brain microsomes were carried out as previously described (4,5).

RESULTS

Microsomes isolated from whole rat brain microsomes contained measurable levels of cytochrome P-450 ranging from 0.025 to 0.051 nmoles/mg with a mean of 0.036 nmoles/mg (Table I). The NADPH cytochrome c reductase activity ranged from 26 to 55 nmoles of cytochrome c reduced/mg/min with a mean of 38.9 nmoles/mg/min. Microsomes isolated from rat liver contained approximately 30 times the amount of cytochrome P-450 and 4 times the cytochrome c reductase activity present in those from rat brain (Table I).

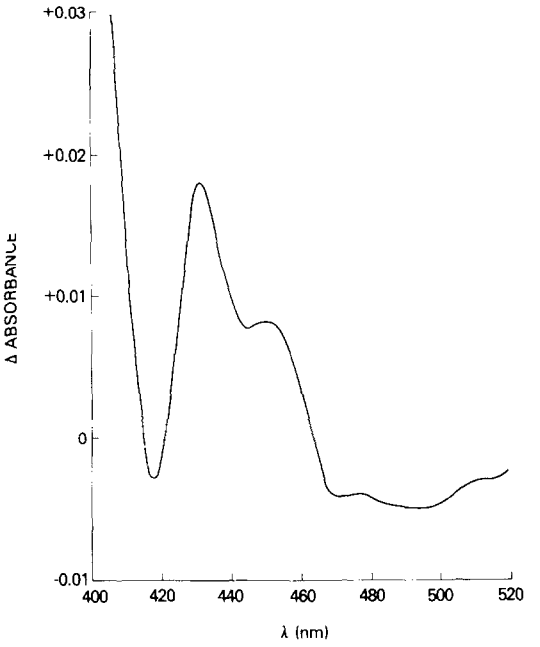

Figure 1: CO-Reduced difference spectrum of rat brain microsomal cytochrome P-450. The spectrum was recorded at a hemoprotein concentration of 80 picomoles/ml as described in Methods.

Thus, the ratio of cytochrome c reductase activity to the amount of cytochrome P-450 is approximately 10 times greater in brain than in liver.

The CO difference spectrum of rat brain microsomes after reduction with NADPH (Figure 1) was virtually identical to that observed after reduction with dithionite. In all cases the relative size of the peak appearing at 430 nm did not influence the location of the 450 nm region peak. Addition of 17β-estradiol in the absence of a reducing agent causes a reverse Type I difference spectrum with rat brain microsomal cytochrome P-450 (Figure 2) and a typical Type I spectrum with rat liver microsomes. Reverse Type I spectra have been observed with other steroids and microsomes isolated from extrahepatic tissues (10).

Microsomes isolated from rat brain catalyzed the oxidation of 17β-estradiol to a reactive metabolite which became covalently bound to

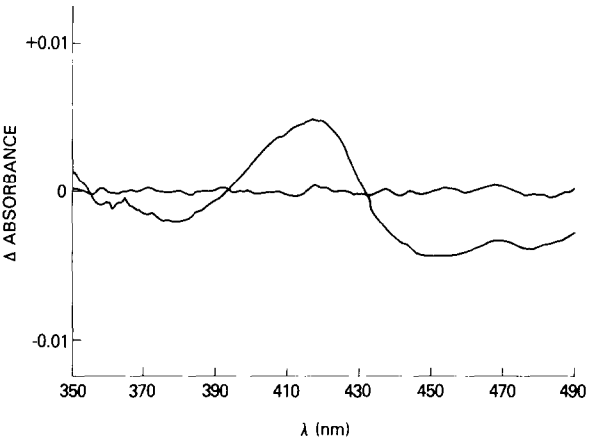

Figure 2: Reverse Type I difference spectrum of rat brain microsomal cytochrome P-450 induced with 17β-estradiol. The spectrum was recorded at a hemoprotein concentration of 80 picomoles/ml and a substrate concentration of 0.3mM.

microsomal protein. This irreversible binding of radiolabeled estradiol was observed in the presence of air and NADPH and was almost abolished by heat denaturation of the microsomal enzyme or by anaerobiosis or the omission of NADPH (Table IIA). An atmosphere of $CO:O_2$(9:1) decreased the binding by 63% and an antibody against NADPH cytochrome c reductase decreased it by approximately the same amount. Superoxide dismutase decreased NADPH-dependent binding by approximately 20%, similar to results obtained with rat liver microsomes (4).

The catechol estrogen, 2-hydroxyestradiol, was found to bind covalently to microsomal protein to a much greater extent than estradiol (Table IIB). This reaction was also significantly inhibited by either the omission of NADPH or the addition of an antibody against NADPH cytochrome c reductase. However, a $CO:O_2$(9:1) atmosphere had no effect on the binding. In contrast, superoxide dismutase decreased the NADPH-dependent binding by more than 50%.

Substantial covalent binding of epinephrine to rat brain microsomal protein was observed (Table IIIA). This binding reaction was also found to

Table II Conditions for covalent binding of [6,7-^{3}H]- estradiol and [6,7-^{3}H]-2-hydroxyestradiol to rat brain microsomal protein in vitro

Results are means ±S.E. of 8 determinations:

Reaction Mixture	Irreversible Binding
A. Estradiol (0.5mM)	picomoles/mg/10min
Complete (air atmosphere)[a]	26.9 ± 1.87
Microsomes (heated 80° x 10min)	7.58 ± 0.24
- NADPH	5.10 ± 0.08
- O_2 (100% N_2 atmosphere)	7.95 ± 0.68
+ N_2:O_2 (9:1 atmosphere)	26.1 ± 1.25
+ CO:O_2 (9:1 atmosphere)	13.0 ± 0.72
+ Preimmune γ-globulin[b]	28.4 ± 1.11
+ Immune γ-globulin[b](NADPH cytochrome c reductase antibody)	12.1 ± 1.05
+ Superoxide Dismutase(15ug/ml)	21.8 ± 0.70
B. 2-Hyrdroxyestradiol (0.5mM)	nmoles/mg/10min
Complete (air atmosphere)[a]	21.4 ± 1.44
- NADPH	4.09 ± 0.23
+ CO:O_2 (9:1 atmosphere)	19.7 ± 0.94
+ Preimmune γ-globulin[b]	23.0 ± 0.75
+ Immune γ-globulin[b]	9.2 ± 1.00
+ Superoxide Dismutase (15ug/ml)	12.6 ± 0.84

a. Incubation mixture with a NADPH generating system as described in Methods.
b. Incubation were as described in Methods.

require NADPH, oxygen and microsomes. Covalent binding was inhibited by an antibody against NADPH cytochrome c reductase but not by a CO:O_2(9:1) atmosphere. Superoxide dismutase and ascorbic acid almost abolished the reaction, whereas catalase and sodium benzoate had no effect. Other radiolabeled catecholamines were also found to bind covalently to rat brain microsomal protein (Table IIIB). In contrast, a methylated catechol was not significantly bound.

DISCUSSION

The present studies show that rat brain microsomes contain measurable levels of cytochrome P-450. This cytochrome is similar to hepatic cytochrome P-450 in that it is required for the oxidation of estradiol to a

Table III Conditions for the covalent bindings of radiolabeled catecholamines[a] to rat brain microsomal protein

Results are means ± S.E of 8 determinations

Reaction Mixture	Irreversible Binding
A. Epinephrine (0.5mM)	nmoles/mg/10min
Complete (air atmosphere)[b]	5.91 ± 0.31
Microsomes (heated 80°x10 min)	0.87 ± 0.05
- NADPH	0.51 ± 0.10
- O_2	0.57 ± 0.03
+ N_2:O_2 (9:1 atmosphere)	4.91 ± 0.27
+ CO:O_2 (9:1 atmosphere)	5.40 ± 0.36
+ Preimmune γ-globulin[c]	6.13 ± 0.48
+ Immune γ-globulin[c]	2.05 ± 0.32
+ Superoxide Dismutase (15μg/ml)	0.61 ± 0.07
+ Ascorbic acid (0.5mM)	0.84 ± 0.07
+ Catalase (0.1mg/ml)	5.36 ± 0.34
+ Sodium benzoate (5mM)	5.69 ± 0.54
B. Other Catechols (0.5mM)	
Norepinephrine	5.94 ± 0.19
Dopamine	5.64 ± 0.45
α-Methyl-L-Dopa	3.96 ± 0.05
L-Dopa	4.49 ± 0.09
3-)-Methyl-L-Dopa	0.77 ± 0.07

a. See the reference 9, 10 for the position of the radiolabel.
b. See Table II[a]
c. See Table II[b]

reactive metabolite that binds covalently to tissue protein (4,11). The NADPH cytochrome c reductase activity present in rat brain microsomes was inhibited by an antibody prepared against hepatic microsomal NADPH cytochrome c reductase. The brain enzyme was also found to be necessary for the oxidation of estradiol to a reactive metabolite.

The reaction sequence for the oxidation of estrogens to metabolites which bind to microsomal protein has been postulated to occur in two steps: 1) the oxidation of estrogens to 2-hydroxyestrogens followed by 2) the oxidation of the catechol to a semiquinone (12). Using the COMT assay procedure described by Paul et al (3) we have found that the antibody against NADPH cytochrome c reductase decreased the formation of 2-hydroxyestradiol from

35.6 ± 1.79 to 11.6 ± 0.77 picomoles/mg/min in rat brain microsomes. The reductase in brain microsomes is also important in the conversion of catechols to metabolites which bind to microsomal protein as shown by the inhibition of the covalent binding of 2-hydroxyestradiol (Table IIB) and epinephrine by the antibody (Table IIIA). Moreover, the covalent binding of the catechol is considerably decreased by superoxide dismutase and unaffected by catalase or sodium benzoate, suggesting that superoxide may be required for the formation of the reactive metabolites. However, in contrast to previous results obtained on the covalent binding of 2-hydroxyestrogens (4) and the metabolism (13) and covalent binding (14,15) of catecholamines to rat liver microsomes, the binding of 2-hydroxyestradiol and epinephrine to rat brain microsomes is not inhibited by a $CO:O_2$(9:1) atmosphere. Thus, the major source of superoxide in brain is not cytochrome P-450 but NADPH cytochrome c reductase, which is also known to generate superoxide anions (15).

Whether or not the reactions observed with rat brain microsomes play a role in either the antiestrogenic effect of 2-hydroxyestradiol (16) or the toxicological effects of related compounds, such as 6-hydroxydopamine (17), is unclear at this time. However, the presence of measurable levels of cytochrome P-450 and NADPH cytochrome c reductase activity in the brain is intriguing since this organ is a target tissue for many drugs and hormones.

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