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Lanosterol 14-Demethylase Activity Expressed in Rat Brain Microsomes[1]

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The occurrence of sterol 14-demethylase in rat brain microsomes was confirmed. The brain microsomes from adult rats converted lanosterol into its 14-demethylated products, 4,4-dimethylcholesta-8,24-dienol, and 4,4-dimethylcholesta-8,14,24-trienol, in the presence of NADPH and molecular oxygen. This metabolism of lanosterol was inhibited by carbon monoxide and ketoconazole, a potent inhibitor of sterol 14-demethylase P450 (P45014DM or CYP51). These facts indicated the occurrence of lanosterol 14-demethylation in rat brain microsomes and its dependency on P45014DM. A representative value of the lanosterol demethylase activity of the brain microsomes was 8.4 pmol/min/mg protein or 640 pmol/min/nmol of total P450. The former was about one-thirteenth of the corresponding value observed with liver microsomes from the same rats, while the latter was 4-times higher than the corresponding value obtained with the liver microsomes. This fact suggested that the ratio of P45014DM to other P450 species was higher in brain than in liver. Lanosterol 14-demethylation is situated at the root of the sterol-biosynthetic branch of the mevalonic acid pathway. Therefore, the present finding enzymologically supports the existence of the sterol biosynthetic pathway in brain.

Key words: brain, cholesterol, P450, sterol synthesis, sterol 14-demethylase.

The brain contains a large amount of cholesterol as an essential component of nerve cell membranes. Cholesterol may also serve as the starting material for the biosynthesis of brain steroid hormones or neurosteroids, since several papers (*1-9*) have suggested the occurrence of enzymes participating in the bioconversion of cholesterol into steroid hormones in brain. Brain cholesterol was reported to be synthesized *in situ* (*10*), and Ramsey *et al.* (*11, 12*) demonstrated that rat brain converted sterol precursors, such as mevalonate and lanosterol into cholesterol and its 4-methylated precursors. On the other hand, there is significant exchange of cholesterol between brain and plasma (*13, 14*), and the meaning of the *in situ* cholesterol synthesis in brain is not yet clear. Examination of the activity and expression level of the enzymes participating in the cholesterol biosynthesis in brain is necessary. Expression of HMG-CoA reductase, the principal rate-limiting enzyme of sterol biosynthesis, in brain has been reported (*15*). However, the reaction catalyzed by this enzyme is situated at the root of the mevalonic acid pathway and is responsible for the production of other functional isoprenoids. Therefore, the activity or expression level of this enzyme is not always taken as an exact index of sterol biosynthesis.

Lanosterol is the initial steroidal precursor in the sterol-biosynthetic branch of the mevalonic acid pathway. According to Ramsey *et al.* (*11*), about half of the radioactivity incorporated into sterols from ^{14}C-mevalonate injected into adult rat brain was recovered in lanosterol. It was also reported (*12*) that 40% of the radioactivity of ^{14}C-mevalonate incubated with developing rat brain homogenate was accumulated in lanosterol. These facts seem to suggest the existence of a rate-limiting step early in the metabolism of lanosterol in brain. The initial reaction in the metabolism of lanosterol to cholesterol by mammals is the 14-demethylation catalyzed by sterol 14-demethylase P450 (P45014DM or CYP51) (*16-18*). Accordingly, P45014DM is one of the important objects for enzymatic investigation of brain sterol synthesis. This paper presents evidence showing the existence of P45014DM-dependent lanosterol 14-demethylation in rat brain microsomes, together with a comparison of the brain and hepatic activities.

MATERIALS AND METHODS

Preparation of Rat Brain Microsomes—The brains of untreated adult Sprague-Dawley rats were homogenized with 3 volumes of 0.1 M potassium phosphate buffer (pH 7.5) in a Potter homogenizer after a brief disruption with a Polytron. The homogenate was centrifuged at 10,000×*g* for 15 min, and the supernatant was further centrifuged at 100,000×*g* for 90 min. The microsomal fraction, recovered as the pellet of the second centrifugation, was suspended in 0.1 M potassium phosphate buffer (pH 7.5). The microsomes and the cytosol separated from the microsomes by the second centrifugation were stored at −80°C under

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Abbreviation: P45014DM; sterol 14-demethylase P450 (CYP51).

nitrogen gas until use.

Assay of Lanosterol 14-Demethylase Activity—Lanosterol 14-demethylase activity was assayed according to the previous method (*19*) except that the enzyme preparation and the cytosolic fraction added to enhance the demethylase activity were replaced with 2 mg protein of the brain microsomes and 10 mg protein of the brain cytosol, respectively, and 50 μM lanosterol (97% pure, Sigma Chemical) dispersed with Tween 80 (50 mg/mg lanosterol) was used as the substrate. The reaction was carried out aerobically at 37°C for 30 min and terminated by adding 5 ml of 10% KOH/methanol. After 60 min saponification at 80°C, sterols in the reaction mixture were extracted with diethyl ether/petroleum ether, and analyzed with GLC (*19, 20*). The demethylase activity was calculated from the gas-chromatographically determined conversion ratio of lanosterol to the demethylated products, as described previously (*20*).

Other Methods—P450 content was determined by second-derivative spectrophotometry. The calibration line was obtained with the dithionite-reduced CO-complex of rat liver microsomal P450. Absolute absorption spectra of dithionite-reduced and CO-saturated suspensions containing different amounts of liver microsomes were recorded with a Shimadzu UV-300 recording spectrophotometer equipped with a head-on type detector, and were converted into the second-derivative spectra by a Shimadzu SAPCOM-1 spectral-data processor using a $d\lambda$ value of 1.2 nm. The signal-strength differences of the derivative spectra [$\Delta(d^2A/d\lambda^2)$] between 450 and 462 nm were plotted against the P450 concentrations of the suspensions calculated from the P450 content of the microsomes and the amount of microsomes in each suspension. The calibration line showed good linearity (Fig. 1) and the slope was not affected by turbidity of the specimen. The second-derivative spectrum of the brain microsomes was recorded under the same conditions as above, and its P450 content was calculated by using the calibration line. NADPH-P450 reductase activity was assayed by the method of Yasukochi and Masters (*21*). Protein was determined by the method of Lowry *et al.* (*22*), by using bovine serum albumin as the standard.

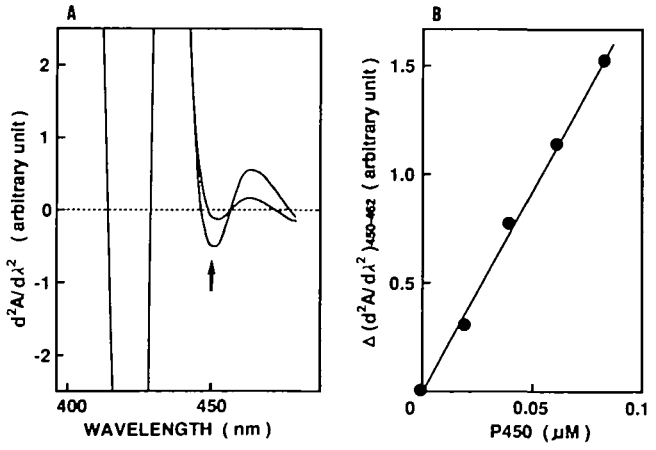

Fig. 1. **Typical second-derivative spectra of the dithionite-reduced and CO-saturated rat liver microsomes (A) and the calibration line for P450 determination by second-derivative spectrophotometry (B).** A: Suspensions of rat liver microsomes containing 0.02 and 0.05 μM P450 were reduced with dithionite and saturated with CO. The absolute spectra of these suspensions were recorded and their second-derivative spectra ($d^2A/d\lambda^2$ against λ) were depicted by using a $d\lambda$ value of 1.2 nm. The second-derivative signal of the Soret peak of the reduced CO-complex of P450 is indicated by an arrow. B: The signal strength difference [$\Delta(d^2A/d\lambda^2)$] between the trough (450 nm) and the peak (462 nm) was plotted against the P450 concentration in the suspension.

RESULTS AND DISCUSSION

Lanosterol 14-Demethylase Activity of the Rat Brain Microsomes—Aerobic incubation of lanosterol with the rat brain microsomes under the conditions described in "MATERIALS AND METHODS" gave two sterols, detected as peaks P1 and P2 in the gas-chromatogram (Fig. 2B). They were not observed in the gas-chromatogram of sterols extracted at zero time of the incubation (Fig. 2A). Relative retention times of P1 and P2 to peak S (lanosterol) correspond, respectively, to those of 4,4-dimethylcholesta-8,24-dienol and 4,4-dimethyl-cholesta-8,14,24-trienol produced by the

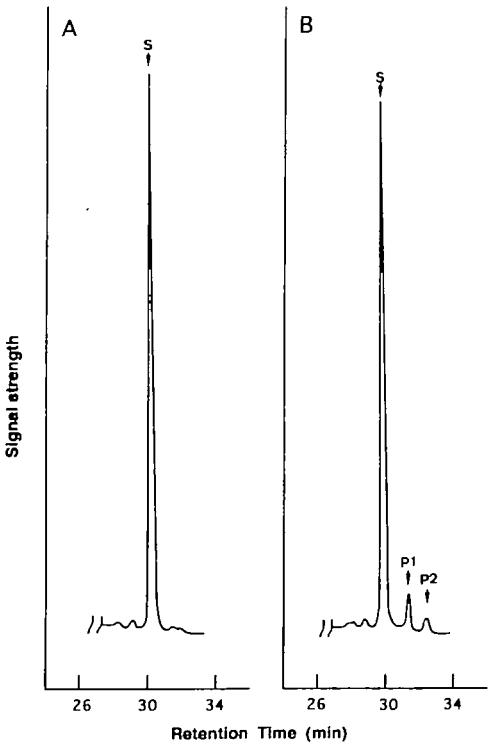

Fig. 2. **Gas-chromatograms of sterols extracted from the reaction mixture before (A) and after (B) incubation of lanosterol with rat brain microsomes.** Lanosterol (20 nmol) was incubated aerobically at 37°C for 90 min with 1.6 mg protein of rat brain microsomes in the presence of 11.6 mg of rat brain cytosol and an NADPH generating system. The reaction mixture was saponified, sterols were extracted and the lanosterol fraction was separated by silica-gel TLC (*20*). The lanosterol fraction extracted from the TLC plate was trimethylsilylated (*20*) and subjected to gas-chromatography with a DB-17 fused-silica capillary column at 255°C. Peak S was lanosterol and peaks P1 and P2 were the products formed from lanosterol. The products, P1 and P2, were identified as 4,4-dimethylcholesta-8,24-dienol and 4,4-dimethylcholesta-8,14,24-trienol, respectively, by comparing their relative retention times to lanosterol with those of authentic specimens prepared by the 14-demethylation of lanosterol with yeast microsomal lanosterol 14-demethylase (*20*).

14-demethylation of lanosterol by yeast microsomal lanosterol 14-demethylase (*20*) to lanosterol determined under the same conditions. The formation of these two peaks was not observed when NADPH was omitted from the reaction medium. These facts clearly indicated the occurrence of NADPH-dependent aerobic conversion of lanosterol to the 14-demethylated products in rat brain microsomes.

The total amount of the 14-demethylated products of lanosterol was calculated from the gas-chromatographically determined conversion ratio (sum of the peak areas of P1 and P2 over sum of peak areas of P1, P2, and S in Fig. 2B) and the amount of lanosterol added to the incubation mixture, as described previously (*20*). As shown in Fig. 3, the sum of the amount of these products increased with time. Lanosterol 14-demethylase activity of the brain microsomes assumed from the initial rate was 6.2 pmol/min/mg protein. Since the recovery of the microsomes was 5.22 mg protein/g tissue (Table II), the apparent lanosterol 14-demethylase activity of the brains was assumed to be 32.4 pmol/min/g tissue. Ramsey *et al.* (*12*) reported that 6 h incubation of ^{14}C-lanosterol (0.26 μmol, 1.2 μCi) with developing (15-day-old) rat brain homogenate corresponding to 3 g of tissue gave $1,300 \times 10^3$ dpm of ^{14}C-labeled nonsaponifiable substances and 24% of the radioactivity was recovered as digitonides. Assuming the radioactivity recovered in the digitonides was attributable to the sterols derived from the ^{14}C-lanosterol, the apparent activity of lanosterol metabolism by the brain homogenate was roughly estimated to be 30 pmol/min/g tissue. This value is comparable to the lanosterol 14-demethylase activity described above, although the ages of the rats used in the studies were different.

Table I summarizes the cofactor requirement and typical results of inhibition experiments on lanosterol 14-demethylase activity of the brain microsomes. No demethylase activity was observed when NADPH was omitted from the reaction mixture. The demethylase activity was sensitive to CO and was completely inhibited by ketoconazole (15 μM), a potent inhibitor of P45014DM (*23, 24*). The inhibitory effect of ketoconazole on the brain demethylase was comparable to that on rat liver P45014DM (*24*), but was lower than that on the yeast enzyme (*25*). These facts indicated that lanosterol 14-demethylation occurring in the rat brain microsomes is catalyzed by P45014DM. However, the inhibitory effect of CO was incomplete (48%) even under a high partial pressure of CO ($CO : O_2 = 95 : 5$), and was lower than that on yeast P45014DM (*20*).

The brain lanosterol 14-demethylase activity was reduced to 61.4% of that of the complete system when the brain cytosol fraction was omitted from the reaction system (Table I). Enhancement of lanosterol 14-demethylase activity by cytosolic fraction was observed with liver microsomes (*19, 26*), and this effect of cytosol was considered to be due to the presence of a putative carrier protein promoting the access of lanosterol to the active site of P45014DM in the microsomal membrane. Accordingly, the above finding suggested that P45014DM of the rat brain microsomes also required a similar cytosolic carrier for maximum activity. It can thus be concluded that rat brain microsomes contain essentially the same lanosterol 14-demethylase system as that of liver microsomes. Recently, we (*27, 28*) reported that P45014DM was the most conserved eukaryotic P450 species thus far known, and in mammals, this P450 might be expressed even in germ line cells (*28*). Accordingly, P45014DM expressed in brain is likely to be the same molecular species as that expressed in liver.

Contents of P450, NADPH-P450 Reductase and Lanosterol 14-Demethylase—The P450 content of the brain microsomes was determined by second-derivative spectrophotometry, as described in "MATERIALS AND METHODS." Although little absorption was observed either in the reduced-CO difference spectrum of the brain microsomes (data not shown) or in the absolute spectrum of the dithionite-reduced and CO-saturated brain microsomal suspension (Fig. 4A), a distinct trough corresponding to the Soret peak of the reduced CO-complex of P450 could be observed at 450 nm in the second-derivative spectrum of the latter (Fig. 4B). The P450 content of the brain microsomes calculated from the second-derivative spectrum with the calibration line (Fig. 1) was 0.018 nmol/mg protein. NADPH-P450 reductase activity of the brain microsomes expressed as cytochrome *c* reducing activity was 0.035 μmol/min/mg protein. These values agree well with the reported contents of these enzymes in rat brain microsomes (*29-31*).

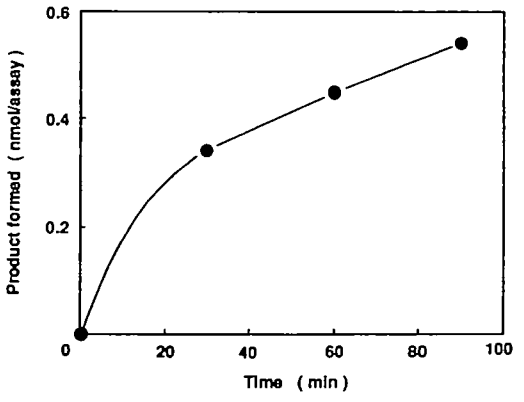

Fig. 3. **A typical time course of lanosterol 14-demethylation by the rat brain microsomes.** Lanosterol 14-demethylase activity was assayed as described in "MATERIALS AND METHODS" by using 2.0 mg protein of microsomes and 10.0 mg protein of cytosol. The ordinate shows the sum of the amounts of 4,4-dimethylcholestatrienol and 4,4-dimethylcholestadienol formed from lanosterol.

TABLE I. **Co-factor requirement of the brain microsomal lanosterol 14-demethylase activity and the inhibitory effects of ketoconazole and carbon monoxide.** Lanosterol 14-demethylase activity was assayed as described in "MATERIALS AND METHODS." The activities of "Complete" and "Control" were 10.0 and 8.1 pmol/min/mg protein, respectively. Ketoconazole was added as 10 μl of dimethylsulfoxide solution and an equal volume of the solvent was added to "Control." The activity observed under $N_2 : O_2 = 95 : 5$ was 8.4 pmol/min/mg protein.

Reaction system	Relative activity (%)
Complete	100
−NADPH	0
−Cytosol	61.4
Control	100
+Ketoconazole (15 μM)	0
$N_2 : O_2 = 95 : 5$	100
$CO : O_2 = 95 : 5$	52

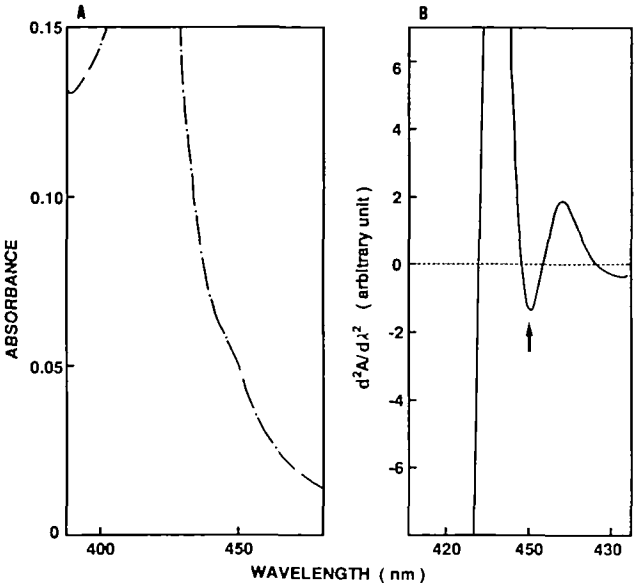

Fig. 4. **Absolute and second-derivative spectra of the dithionite-reduced and CO-saturated suspension of the rat brain microsomes.** The rat brain microsomes (8.6 mg protein) were suspended in 2.5 ml of 0.1 M potassium phosphate buffer, pH 7.5. The suspension was gently bubbled with CO for 30 s and reduced with dithionite, and the absorption spectrum was recorded against a suspension of skim milk showing nearly the same turbidity as the microsomal suspension. B: The second-derivative spectrum of A obtained under the same conditions as Fig. 1. The arrow indicates the second-derivative signal due to the Soret peak of the reduced CO-complex of P450.

TABLE II. **Comparison of the lanosterol 14-demethylase activity, P450 content, and NADPH-P450 reductase activity between brain and liver microsomes of rats.**

Organ	Yield of microsomes (mg prot/g organ)	Lanosterol 14-demethylase (pmol/min/mg prot)	Lanosterol 14-demethylase (pmol/min/nmol P450)	P450 (nmol/mg prot)	NADPH-P450 reductase (μmol cyt.c/min/mg prot)
Brain	5.22	8.40	640	0.018	0.035
Liver	29.2	110	160	0.75	0.15

Table II shows a comparison of the lanosterol 14-demethylase activities of the brain and the liver microsomes prepared from the same rats, together with their P450 content and P450 reductase activity. The lanosterol 14-demethylase activity and the P450 content of the brain microsomes were about one-thirteenth and one-fortieth, respectively, of those of the liver microsomes. Consequently, the lanosterol 14-demethylase activity per P450 was four times higher in the brain than the liver, indicating that the ratio of P45014DM to other P450 species was higher in the brain than in the liver. Based on the highest activity reported for a purified preparation of rat liver lanosterol 14-demethylase (9.4 nmol/min/nmol P450) (*28*) and the lanosterol demethylase activity shown in Table II, 5–10% of the brain P450 may correspond to P45014DM. NADPH-P450 reductase activity was also lower in the brain than in the liver, but the activity ratio was smaller than that of P450, indicating that the lower lanosterol 14-demethylase activity of the brain microsomes was due not to a shortage of the reductase, but to the low content of P45014DM.

General Discussion—The results described in this paper clearly demonstrate the existence of P45014DM-dependent lanosterol 14-demethylase activity in rat brain microsomes. Since lanosterol 14-demethylation is situated at the root of the sterol-biosynthetic branch of the mevalonic acid pathway, this finding may be taken as evidence indicating the occurrence of the typical sterol biosynthetic pathway in brain. As reported by Ramsey *et al.* (*11*), a rat brain cell-free preparation converted mevalonic acid into sterols, and expression of HMG-CoA reductase in brain has been reported (*15*). It can thus be concluded that cholesterol is synthesized *de novo* in brain. Based on the lanosterol 14-demethylase activity described in this paper and the result of the experiment by Ramsey *et al.* (*12*) on ^{14}C-lanosterol metabolism by the rat brain homogenate, the sterol biosynthetic activity of brain is lower than that of liver. However, the apparent low activity observed in the microsomes isolated from the whole-brain homogenate or the brain homogenate itself does not necessarily imply a low cellular content of the enzyme, and there may be a possibility that lanosterol 14-demethylase is locally expressed in specific cells in brain. If this is the case, it is likely that the cholesterol synthesized *de novo* in brain has some specific functions, *e.g.* as a source material for neurosteroids acting in a paracrine manner. Some steroidogenic P450s have been reported to be expressed in limited regions in brain (*2, 5–9*). We are going to perform histochemical examination of brain P45014DM by using P45014DM cDNA (*27, 28*) and antibodies against this P450 (*27*).

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