HEPATIC AMINOPYRINE N-DEMETHYLASE SYSTEM:
EFFECT OF CYANIDE ON MICROSONAL N-DEMETHYLASE ACTIVITY

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Abstract—Cyanide, an inhibitor of many hemoproteins, was shown to affect a number of microsomal drug-metabolizing activities catalyzed by cytochrome P-450. The N-demethylation reaction of aminopyrine was inhibited noncompetitively by this inhibitor in microsomal preparations from rats. The binding reaction of aminopyrine with microsomal cytochrome P-450 was also modified by cyanide, and an abnormal aminopyrine-induced difference spectrum of microsomes appeared when cyanide was added to the reaction mixture. Partial dissociation of cytochrome P-450-cyanide complex by aminopyrine was observed by spectrophotometrical and epr spectroscopic methods. These results suggest that aminopyrine and cyanide reciprocally affect binding with cytochrome P-450 and modification by cyanide of aminopyrine binding reaction with the hemoprotein produces an inhibition of N-demethylase activity.

Cytochrome P-450 is known to be a terminal oxidase of microsomal monooxygenase system associated with the oxidative biotransformation of both endogenous and exogenous substances (1-3). The hydroxylation reaction catalyzed by this hemoprotein is inhibited by various compounds, such as CO, SKF 525-A, metyrapone and ethylisocyanide, etc. These inhibitors are also known to bind with cytochrome P-450, accompanied by spectral perturbation of the hemoprotein (4-7). Cyanide is a potent inhibitor of many hemoproteins, and its binding with microsomal cytochrome P-450 has been also reported by several investigators (8-11). However, liver microsomal hydroxylase activity is generally considered to be insensitive to cyanide (12-14).

In previous papers, we reported that microsomal aniline hydroxylase activity is inhibited markedly by cyanide (15, 16). Since almost all substrates for the microsomal hydroxylase system are classified as type I compounds and aniline is the only known substrate among type II compounds (8), the effect of cyanide on the oxidative biotransformation of type I substrates was examined and compared to that on the aniline hydroxylase system.

MATERIALS AND METHODS

Male Wistar rats weighing 250-300 g, were given phenobarbital in 0.9% NaCl (40 mg/kg, i.p.) for three days. Liver microsomes were prepared as described previously (17), and microsomal protein concentration was determined by the biuret reaction (18) using bovine serum albumin as standard.

The activities of microsomal N-demethylase and aniline hydroxylase were determined
as described previously (15, 17). The aliphatic hydroxylase activities of hexobarbital and
cyclophosphamide were measured at 20°C by determining the initial rate of NADPH oxi-
dation (19). The reaction was initiated by adding 0.5 μmoles NADPH to 3 ml of the
reaction mixture consisting of 50 mM Tris-HCl buffer (pH 7.4), 150 mM KCl, 10 mM MgCl2,
0.4 mg protein of microsomes and 2 mM substrate, and the decrease of the absorption
intensity at 340 nm due to the oxidation of NADPH was recorded. NADPH concentration
was calculated using the extinction coefficient at 340 nm of 6.22 × 10³ M⁻¹ cm⁻¹ (20).

Difference spectra of microsomal cytochrome P-450 were determined using a Shimadzu
UV-300 spectrophotometer. Epr spectroscopy was performed as described previously
(16) using a Varian V-4502-15 X-band spectrometer with a 100 KHz magnetic field modu-
lation unit.

Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP and NADPH were
purchased from Oriental Yeast Co. (Osaka); sodium phenobarbital, from E. Merck Ag.
(Germany); aminopyrine, from Iwaki Seiyaku Co. (Tokyo); cyclophosphamide and sodium
hexobarbital, from Shionogi & Co. (Osaka); and ethylmorphine and methamphetamine,
from Dainippon Seiyaku Co. (Osaka). Aniline was distilled under reduced pressure and
the purity checked by gas chromatography. Potassium cyanide solutions were prepared
as needed and carefully adjusted to pH 7.4 with hydrochloric acid just prior to use. Other
chemicals of the highest quality available were obtained commercially and were not purified
further.

RESULTS
Effect of cyanide on the formation of formaldehyde from aminopyrine by liver microsomes

N-Demethylation of various compounds is a typical reaction of microsomal mono-
oxynogenase system, and aminopyrine is probably one of the most widely employed substrates
for the microsomal N-demethylase system (21). The reaction is catalyzed by cytochrome
P-450 (13), and the substrate aminopyrine is classified as a type I substrate (8). Thus, the
effect of cyanide on the oxidative biotransformation of type I substrates was studied using
aminopyrine as a model compound. The time course of formaldehyde production from
aminopyrine by microsomal enzymes was linear for about 2–3 min, then the rate decreased
with time (17). Addition of cyanide to the incubation mixture resulted in a marked decrease
of formaldehyde production (Fig. 1). Formaldehyde, which is the reaction product of
aminopyrine N-demethylation, is known to form cyanohydrin by addition reaction of
hydrogen cyanide. However, a known amount of formaldehyde with or without 10–20 mM
cyanide was carried through the incubation and assay procedure, and the absorbance inten-
sity of chromogen in the resultant solution was determined to be the same regardless of
the presence of cyanide in the assay medium. In addition, when aminopyrine N-deme-
thylase activity was measured in the incubation system containing semicarbazide as the
trapping agent of formaldehyde produced, the activity was also inhibited by cyanide. There-
fore, the inhibitory action of cyanide on aminopyrine N-demethylase system of microsomes
had to be considered. The effect of cyanide on microsomal N-demethylase activity was
FIG. 1. Time course of formaldehyde production from aminopyrine by rat liver microsomes. Microsomal aminopyrine N-demethylase activity was measured in 2.5 ml of 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM aminopyrine, 150 mM KCl, 10 mM MgCl₂, 0.36 mM NADP, 4 mM glucose-6-phosphate, 1 unit of glucose-6-phosphate dehydrogenase and 2.5 mg protein of liver microsomes from phenobarbital-pretreated rats. The reaction was initiated by adding microsomes to the reaction mixture and carried out at 37°C. Open and closed circles indicate the absence or presence of 5 mM cyanide, respectively.

FIG. 2. Inhibition of aminopyrine N-demethylase activity by cyanide. The N-demethylase activity of aminopyrine was estimated in the reaction mixture described in Fig. 1. Microsomes prepared from normal rats were used instead of those from phenobarbital-pretreated rats. Cyanide concentrations in the reaction mixture were (a) 0, (b) 1 or (c) 5 mM.

FIG. 3. Lineweaver-Burk plot indicating the effect of cyanide on the velocity of aminopyrine N-demethylation. The data of Fig. 2 were plotted as the reciprocal of initial reaction velocity versus the reciprocal of molar concentration of aminopyrine. Cyanide concentrations were (a) 0, (b) 1 or (c) 5 mM. The inset is a Hill plot of aminopyrine N-demethylation. Open and closed circles indicate the absence or presence of 5 mM cyanide, respectively.
then examined under such conditions of linearity.

Fig. 2 (curve a), showing the relationship between N-demethylase activity and the concentration of aminopyrine, is a Michaelis-Menten type curve. A double reciprocal plot (Fig. 3, curve a) of the activity versus substrate concentration, suggests the existence of two species of cytochrome P-450 associated with the N-demethylation reaction. Michaelis constants for these two reaction phases were estimated graphically to be 55 and 670 μM, respectively. The data were then arranged in a Hill plot to obtain the Hill coefficients. The coefficients of the two reaction phases were 1.05-1.10, suggesting that one molecule of aminopyrine could bind to one molecule of cytochrome P-450 during the N-demethylation reaction (Fig. 3, inset).

Addition of cyanide to the reaction mixture resulted in a decrease of N-demethylase activity depending upon cyanide concentration (Fig. 2, curves b and c). The Lineweaver-Burk plot shows that cyanide noncompetitively inhibits both phases of the reaction (Fig. 3), and the inhibitor constants of cyanide for two reaction phases were 1.5 and 5.9 mM, respectively. The Hill plot shows almost the same pattern even in the presence of cyanide, suggesting non-cooperativity of cyanide for the aminopyrine-binding site of cytochrome P-450 (Fig. 3, inset).

Effect of cyanide on aminopyrine-induced spectral change of microsomes

Inhibitory action of cyanide on microsomal aniline hydroxylase system was determined to be the modification of the substrate-binding reaction with cytochrome P-450, accompanied by inhibition of hydroxylase activity (16). Thus, the effect of cyanide on aminopyrine-binding reaction with cytochrome P-450 was studied by spectrophotometrical methods. A normal type I binding spectrum exhibiting a peak and a trough at 385 and 420 nm, respectively, was observed by adding aminopyrine to the microsomal suspension (Fig. 4, spectrum 1). When 1 mM cyanide was added to the contents in both sample and reference cuvettes, an abnormal aminopyrine-induced difference spectrum was obtained as shown in Fig. 4 (spectrum 2). The wavelengths of peak and trough of aminopyrine-induced difference spectrum shifted to long wavelength, at 390 and 438 nm, respectively, and a shoulder appeared at about 420 nm. By increasing concentrations of cyanide, the magnitude of the absorbance change between peak (at about 390 nm) and trough (at about 438 nm) decreased gradually, suggesting that the binding reaction of aminopyrine with cytochrome P-450 was inhibited by cyanide. Of interest was the appearance of another peak and trough at about 416 and 400 nm, respectively (Fig. 4, spectra 2c). Since the wavelength of the trough (438 nm) was close to the peak position (444 nm) of the cyanide-induced difference spectrum of microsomes (Fig. 5, solid line curve), it was presumed that the complex of cytochrome P-450 and cyanide in the sample cuvette would dissociate by adding aminopyrine to the sample cuvette only, accompanied by the appearance of an inverted cyanide-induced difference spectrum (λ_max at 402 nm and λ_min at 444 nm). Spectral participation of the inverted cyanide-induced difference spectrum to the aminopyrine-induced spectral change was thus presumed to cause an appearance of the abnormal difference spectra.

To clarify the nature of the abnormal aminopyrine-induced spectral change (Fig. 4),
FIG. 4. Aminopyrine-induced difference spectra of microsomes. Liver microsomes prepared from normal rats were suspended at a concentration of 2 mg protein/ml (2.61 nmoles cytochrome P-450/ml) in 50 mM Tris-HCl buffer (pH 7.4) containing 150 mM KCl and 10 mM MgCl₂ with or without various concentrations of cyanide, and the samples were divided equally between two cuvettes. After recording base line, 3.2 mM aminopyrine was added to the sample cuvette and the resulting spectra recorded. Final concentrations of cyanide were (1) 0, (2) 1, (3) 2.5, (4) 5, (5) 7.5 and (6) 10 mM, respectively.

FIG. 5. Difference spectra of microsomes induced by cyanide and aminopyrine. Hepatic microsomes were diluted to a concentration of 2 mg protein/ml (2.56 nmoles cytochrome P-450/ml) in the medium shown in Fig. 4, and samples were divided equally between two cuvettes. After recording base line, cyanide (0.17 mM at final) was added to the sample cuvette (solid line curve). Next, 1.67 mM aminopyrine was further added to the sample cuvette and the resulting spectrum recorded (dashed line curve). Finally, aminopyrine (1.67 mM) was added to the reference cuvette (dash-dot line curve). A similar spectral experiment with microsomes was further studied. Typical cyanide-induced difference spectrum (λ_max at 444 nm and λ_min at 402 nm) was observed when cyanide was added to the microsomal suspension (Fig. 5, solid line curve). The peak and trough positions in the difference spectrum shifted to 446 and 407 nm, respectively, by further addition of aminopyrine to the cyanide-pretreated microsomes in the sample cuvette. The magnitude of the absorption change decreased significantly, but not in an additive manner, by the addition of both cyanide and aminopyrine to the microsomal suspension (Fig. 5, dashed line curve). Finally, the difference spectrum of microsomes between the microsomal sample containing both cyanide and aminopyrine and the one containing only aminopyrine was recorded by adding aminopyrine to the reference cuvette. The resultant spectrum indicated an abnormal one, which was similar, but not identical, to the cyanide-induced difference spectrum (Fig. 5, dash-dot line curve). These results also suggest the
dissociation of the cytochrome P-450 cyanide complex by aminopyrine.

Epr spectroscopic observation

To confirm the dissociation of cytochrome P-450-cyanide complex by aminopyrine, epr spectra of the low spin form of cytochrome P-450 were observed at liquid nitrogen temperature. The anisotropic epr absorption of microsomal hemoprotein could be observed exhibiting three strong signals with g values of 1.92, 2.25 and 2.42 (Fig. 6, spectrum 1). Upon adding 50 mM cyanide to the microsomal suspension (1.25 μmol of cyanide/mg protein of microsomes, or 0.57 μmol of cyanide/nmol of cytochrome P-450), the epr absorption spectrum (Fig. 6, spectrum 2) showed the existence of both free and cyanide-bound forms of cytochrome P-450, as described previously (16). Further addition of aminopyrine resulted in an increase of the epr absorption signal at g = 2.25 region, which is the characteristic absorption signal of free or aminopyrine-bound form of cytochrome P-450 (Fig. 6, spectrum 3). These results also indicated the dissociation of the cytochrome P-450-cyanide complex by aminopyrine.

Effect of cyanide on cytochrome P-450-catalyzed drug-metabolizing reactions

As described above, microsomal aminopyrine N-demethylase activity was clearly inhibited by cyanide. Thus, all drug-metabolizing reactions catalyzed by cytochrome P-450 containing monooxygenase system probably are sensitive to cyanide. To confirm this, the effect of cyanide on various types of microsomal drug-metabolizing reactions was studied. The substrates used are shown in Table 1, in which only aniline is classified as a type II substrate. All activities tested were inhibited by adding 10 mM cyanide to the reaction mixture. Interestingly, inhibitory rates with cyanide were higher (70-90%) in the reactions of type I substrates, and the results were consistent with the different inhibitory patterns of cyanide on aminopyrine N-demethylase system (Figs. 2 and 3) from that on the aniline hydroxylase system (15, 16). Thus all reactions catalyzed by cytochrome P-450
TABLE 1. Effects of cyanide on microsomal drug-metabolizing activities

| Substrate          | Type of reaction       | Concentration of substrate (mM) | Activity (nmoles/min/mg protein of microsomes) | Inhibition (%) |
|--------------------|------------------------|---------------------------------|-----------------------------------------------|----------------|
| Aminopyrine        | N-Demethylation        | 10.0                            | 21.08, 4.66                                  | 77.9           |
| Ethylmorphine      |                        | 1.0                             | 7.55, 1.57                                   | 79.2           |
| Methamphetamine   |                        | 1.3                             | 2.06, 0.20                                   | 90.3           |
| Hexobarbital       | Aliphatic hydroxylation| 2.0                             | 28.70, 8.00                                  | 72.1           |
| Cyclophosphamide   |                        | 2.0                             | 3.20, 0.80                                   | 75.0           |
| Aniline            | Aromatic hydroxylation | 0.5                             | 0.68, 0.44                                   | 35.3           |

Drug-metabolizing activities of various substrates catalyzed by hepatic microsomes from normal rats were assayed in the presence or absence of 10 mM cyanide by measuring the rates of product formation or NADPH oxidation. The activities of both N-demethylation and aromatic hydroxylation reactions are expressed as “nmoles product produced/min/mg protein of microsomes”, and the metabolic activities of hexobarbital and cyclophosphamide as “nmoles NADPH oxidized/min/mg protein”.

DISCUSSION

Microsomal hydroxylase system containing cytochrome P-450 as a terminal oxidase was generally considered to be insensitive to cyanide. However, all drug-metabolizing activities catalyzed by this hemoprotein were inhibited clearly by cyanide (Table 1), although the inhibitory pattern of cyanide on aminopyrine N-demethylase activity differed slightly from that with aniline hydroxylation activity. Our results suggest that the primary inhibitory site of cyanide is not the common reaction step, or the electron transport reaction from NADPH to cytochrome P-450, in microsomal hydroxylase system, but rather the specific binding reaction of cytochrome P-450 with aminopyrine or aniline (16). Type I substrates for microsomal hydroxylase system, including aminopyrine, are presumed to bind to the substrate-binding site of the cytochrome P-450 molecule, which is close, but not identical, to the oxygen- or CO-binding site. On the other hand, type II compounds ligate directly to the sixth ligand of heme-iron of the hemoprotein, which is the binding site for oxygen or CO (22). Since cyanide is assumed to bind to the sixth ligand of heme-iron of many hemoproteins, it is reasonable (23) that cyanide noncompetitively inhibits the N-demethylation reaction of aminopyrine (Fig. 3). Non-cooperativity of cyanide for the aminopyrine-binding site of cytochrome P-450 (Fig. 3, inset) also supports the above assumption.

Complex formation of cytochrome P-450 with cyanide is determined to be an essential factor for the inhibitory action of cyanide on microsomal hydroxylase system (16). Such a complex formation is presumed to cause a modification of the binding reaction between cytochrome P-450 and aminopyrine, accompanied by inhibition of aminopyrine N-demethylase activity (Fig. 4). On the other hand, binding reaction of aminopyrine with microsomal cytochrome P-450 was shown to contain both type I and type II components (T. Matsubara, unpublished results). The former component in the binding reaction was assumed to participate in the N-demethylation reaction, whereas the latter component
might modify the binding reaction of cyanide with cytochrome P-450 (Fig. 5, dash-dot line curve). Therefore, inhibitory pattern of cyanide on the N-demethylation reaction of aminopyrine differed slightly from that on the binding reaction of aminopyrine with microsomal cytochrome P-450. The binding reaction between cytochrome P-450 and cyanide (or aminopyrine) remains the subject of further study.

Increasing evidence shows that several forms of cytochrome P-450 exist, which may account for the multiple monooxygenase activities in liver microsomes (10, 24–26). Microsomal aminopyrine N-demethylase activity showed two distinct phases with respect to aminopyrine concentration (Fig. 3), suggesting the association of two species of cytochrome P-450 with the reactions (17). Individual species of microsomal cytochrome P-450 may have the ability to bind with aminopyrine and/or cyanide but differ in their relative affinity to these compounds.

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