MOLECULAR PHARMACOLOGY, 12, 322-334

Reduction of Tertiary Amine *N*-Oxides by Liver Microsomal Cytochrome P-450

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(Received May 27, 1975)

SUMMARY

SUGIURA, MASAHIKO, IWASAKI, KAZUHIDE & KATO, RYUICHI (1976) Reduction of tertiary amine *N*-oxides by liver microsomal cytochrome P-450. *Mol. Pharmacol.*, 12, 322-334.

Reduction of tertiary amine *N*-oxides in rat liver was investigated with imipramine *N*-oxide, tiaramide *N*-oxide, and *N,N*-dimethylaniline *N*-oxide as substrates in the presence of NADPH. In hepatocytes most of the activities were located in the microsomal fraction; the activities of other subcellular fractions were low or negligible. NADPH-cytochrome *c* reductase, solubilized by trypsin or subtilisin, showed no *N*-oxide reductase activity by itself. This evidence clearly distinguishes *N*-oxide reductase from nitro and azo reductase activities. The characteristics of *N*-oxide reductase activity associated with cytochrome P-450 are as follows. (a) The activity of *N*-oxide reductase is inhibited by an atmosphere of carbon monoxide. The concentration of carbon monoxide giving apparent 50% inhibition of imipramine *N*-oxide reduction is 1.0 μM. The inhibition of *N*-oxide reductase activity by carbon monoxide is partially reversed by exposure to white light. (b) The activity is also inhibited in the presence of oxygen, with 50% inhibition occurring at an oxygen concentration of 2-3 μM. (c) Kinetic studies show that the K_m values for the reduction of imipramine *N*-oxide, tiaramide *N*-oxide, and *N,N*-dimethylaniline *N*-oxide are on the order of 0.1 mM. (d) *N*-Oxide reductase activity is inhibited by nitro, nitroso, and azo compounds and hydrazide, while it is stimulated markedly by flavins. (e) The results of stoichiometric studies show that the ratio between reduced pyridine nucleotide consumption and tertiary amine formation is 1:1, and therefore it is presumed that the final products are a tertiary amine and water. The results obtained suggest that 2 electrons are transferred directly from ferrous iron of cytochrome P-450 to the *N*-oxide in the reaction. The following observations suggest that *N*-oxide coordinates to heme iron at the sixth position: first, reduction of *N*-oxides was inhibited by oxygen; second, *N*-oxides produced spectral changes with reduced cytochrome P-450; finally, *n*-octylamine and 2,4-dichloro-6-phenylphenoxyethylamine, which form a hemichrome with reduced cytochrome P-450, markedly inhibited *N*-oxide reduction. The close relationship observed between cytochrome P-450 content and *N*-oxide reductase activity in microsomes from phenobarbital-treated rats of either sex and various ages further supports our hypothesis that NADPH-dependent *N*-oxide reduction is catalyzed by cytochrome P-450 of liver microsomes.

INTRODUCTION

Cytochrome P-450 has been identified as a hemoprotein and a terminal oxidase in the microsomal electron transport system (1) and shown to be the key participant in a variety of biochemical reactions in which molecular oxygen is incorporated into an

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organic molecule, in the form of a hydroxyl group, concomitantly with the oxidation of a reduced pyridine nucleotide. Estabrook and collaborators (2, 3) indicated that the cytochrome P-450 (Fe^{++})-substrate-O_2 complex may be an intermediate in this reaction. The same authors detected the formation of a new spectral species in the presence of NADPH during the steady-state oxidation of a type I substrate by hepatic microsomes from phenobarbital-treated rats. An oxygen-activating function and the oxygenated intermediate of cytochrome P-450 from *Pseudomonas putida* have also been established (4).

There is little evidence dealing with the reductive catalytic nature of cytochrome P-450 (5, 6). Gillette and collaborators (7, 8) suggested that nitro and azo groups of some compounds are reduced by cytochrome P-450 in addition to the reduction by NADPH-cytochrome *c* reductase in liver microsomes. Although many publications of *N*-oxide reduction have appeared in recent years, little is known about *N*-oxide reductase activity in liver microsomes (9–12). In a previous report we suggested the involvement of cytochrome P-450 in *N*-oxide reduction of tiaramide *N*-oxide by liver microsomes (13). The present work was undertaken to establish the characteristics and general mechanism of the reduction of tertiary amine *N*-oxides catalyzed by cytochrome P-450 of liver microsomes.

MATERIALS AND METHODS

Animals. Seven-week-old male Sprague-Dawley rats were used unless otherwise specified. Some animals were treated intraperitoneally with phenobarbital (90 mg/kg) daily for 3 days or with 3-methylcholanthrene (40 mg/kg) daily for 2 days. Control rats were injected with 0.9% NaCl or with olive oil. All treated rats were killed 24 hr after the last injection.

Materials. NADP, NADPH (type I), NADH (grade III), FAD, FMN, methyl viologen, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, trypsin, soybean trypsin inhibitor, and cytochrome *c* were purchased from Sigma Chemical Company. Subtilisin was purchased from Teikoku Chemical Industry Company. Tiaramide and tiaramide *N*-oxide were synthesized in our research laboratories. Imipramine and chlorimipramine were kindly donated by Ciba-Geigy, Ltd., Basel. SKF 525-A[2] was kindly donated by Smith Kline & French Laboratories, and DPEA, by Lilly Research Laboratories. All other reagents were of the highest grade commercially available. Imipramine *N*-oxide and *N,N*-dimethylaniline *N*-oxide were synthesized by the methods of Fishman and Goldenberg (14) and Belov and Savich (15), respectively.

Preparation of cellular fractions. For studies of the intracellular distribution of *N*-oxide reductase activity, liver homogenates were fractionated according to Sedgwick and Hübscher (16). In most experiments the perfused livers were homogenized in 4 volumes of a 1.15% KCl solution and liver microsomes were prepared as described previously (13). Hepatic microsomes were essentially free from hemoglobin contamination. Succinate dehydrogenase activity detected in this preparation was less than 0.2 unit/mg/min, and therefore the microsomal fraction was assumed to be almost free from mitochondrial contamination. For the preparation of protease digested microsomes, a suspension of KCl-washed microsomes (10 mg of protein per milliliter) in 0.1 mM phosphate buffer (pH 7.4) containing 25% glycerol was incubated anaerobically at 0° in the presence of purified trypsin or subtilisin for 15 hr (17).

Assay of tertiary amine N-oxide reductase activity. Tertiary amine *N*-oxide reductase activity of rat liver microsomes was assayed by measuring the formation of tertiary amine. The standard assay medium (5 ml) contained 0.1 M phosphate buffer (pH 7.4), 2 mM NADP, 10 mM $MgCl_2$, 10 mM glucose 6-phosphate, 3.0 IU of glucose 6-phosphate dehydrogenase, 25 mg of microsomal protein, and the indicated tertiary amine *N*-oxide at a concentration of 1.0 mM. The incubation was carried out at 37° for 10 min anaerobically.

The anaerobic experiments, under either N_2 or CO atmosphere, were carried

[2] The abbreviations used are: SKF 525-A, β-diethylaminoethyl diphenylpropylacetate HCl; DPEA, 2,4-dichloro-6-phenylphenoxyethylamine HCl.

out in Thunberg vessels. The incubation mixtures were alternately evacuated and flushed with nitrogen or carbon monoxide, which was passed through a deoxygenizer system (7). Tertiary amine *N*-oxide reductase activities were assayed using gas chromatography, and the rate of formation of tertiary amines was measured.

Gas-liquid chromatographic analyses. Imipramine, tiaramide, and *N,N*-dimethylaniline were analyzed by gas-liquid chromatography, using a Shimazu GC-5A instrument equipped with a dual flame ionization detector.

Imipramine was extracted according to Weder and Bickel (18) and separated from other metabolites by gas chromatography on a 1% XE-60 Chromosorb W AW DMCS column (60–80 mesh). The column temperature was 195°, and the injection port temperature, 250°. The carrier gas was N_2 at a flow rate of 45 ml/min.

N,N-Dimethylaniline was extracted with 5 ml of ethyl ether from the aqueous phase (pH 10) and re-extracted with 0.1 N HCl. The acid extract was made alkaline by adding 0.5 ml of 2 N NH_4OH, and benzyl alcohol was added at this stage to serve as an internal standard. *N,N*-Dimethylaniline and benzyl alcohol were finally extracted from the acid phase with 200 μl of dichloromethane, and a portion of the extract was directly injected into the gas chromatogram. A 20% Ucon oil 50 HB-2000 on Celite 545 (60–80 mesh) column was used. The column temperature was 150°, and the injection port temperature, 200°. The carrier gas was N_2 at a flow rate of 47.5 ml/min.

Tiaramide was assayed as described previously (13).

In each experiment a standard of imipramine, tiaramide, or *N,N*-dimethylaniline was carried through the whole separation procedure, and the resulting calibration curves were used for quantification, with chlorimipramine, perphenazine, or benzyl alcohol as the internal standard, respectively.

Enzyme assays. NADPH-cytochrome *c* reductase was assayed by the method of Williams and Kamin (19). NADH-ferricyanide reductase and NADH-cytochrome *c* reductase were assayed by the method of Takesue and Omura (20, 21). The activities of succinate dehydrogenase and glucose 6-phosphatase were determined according to Pennington (22) and Swason (23), respectively.

Analytical methods. Cytochromes P-450 and P-420 were determined from CO difference spectra of dithionite-treated samples as described by Omura and Sato (24). An increment in the extinction coefficient between 424 and 409 nm of 185 cm^{-1} mM^{-1} (1) was employed for the calculation of cytochrome b_5 content. Protein was determined by the method of Lowry *et al.* (25), with bovine serum albumin as standard.

RESULTS

Reduction of tertiary amine N-oxides to tertiary amines. Reduction of tertiary amine *N*-oxides to their corresponding tertiary amines was substantial in the presence of hepatic microsomes and NADPH. Using tiaramide *N*-oxide and rat liver microsomes as substrate and enzyme source, the reduction rate was linear with respect to microsomal protein concentration (Fig. 1). The reductase activity was affected by the partial pressure of oxygen, and the *N*-oxide reduction rate was about 0.2 nmole/mg/min, using air as the gas phase. The optimum pH for *N*-oxide reductase activity in phosphate buffer was about 7.4. NADPH was an efficient cofactor for the microsomal *N*-oxide reductase. NADH also served as an electron donor. The activity of NADH-dependent *N*-oxide reductase was about 28% of that of NADPH-dependent *N*-oxide reductase activity at a 0.4 mM concentration of each coenzyme. At saturating levels (2 mM) of the coenzymes, activity with NADH was about half of that with NADPH. When imipramine *N*-oxide and tiaramide *N*-oxide were used as substrates and incubated anaerobically, imipramine and tiaramide were the sole metabolites detectable. When *N,N*-dimethylaniline *N*-oxide was used as substrate, 10% monomethylaniline was also detected (26).

Tissue and subcellular distribution of NADPH-dependent N-oxide reductase activity. Table 1 shows the tissue distribution

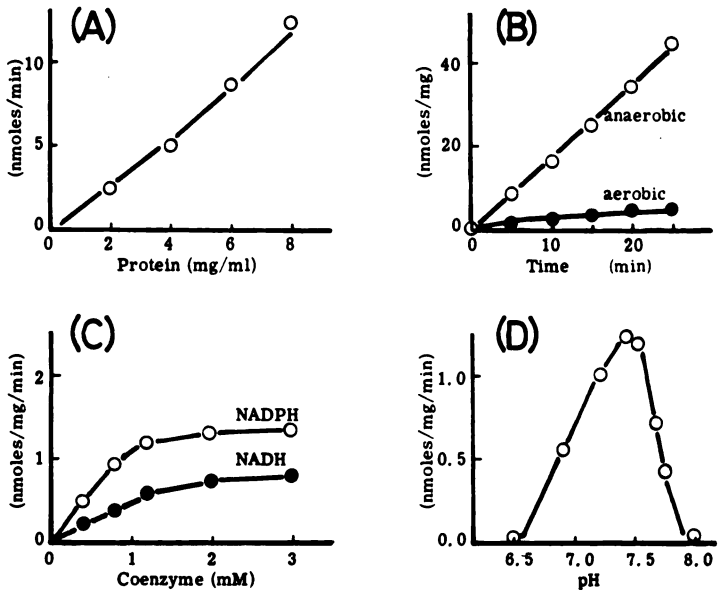


FIG. 1. *Effect of enzyme concentration (A), incubation time (B), NADPH concentration (C), and pH (D) on microsomal NADPH-dependent N-oxide reduction of tiaramide N-oxide*

TABLE 1

Tissue distribution of microsomal NADPH-dependent N-oxide reductase activity

The reaction mixture (5 ml) contained 0.1 M phosphate buffer (pH 7.4), 2 mM NADP, 10 mM $MgCl_2$, 10 mM glucose 6-phosphate, 3.0 IU of glucose 6-phosphate dehydrogenase, microsomal protein equivalent to 1.0 g of tissue (wet weight), and 0.4 mM tiaramide *N*-oxide. The reaction mixtures were incubated at 37° for 10 min anaerobically, and the amount of tiaramide formed was determined.

Source of microsomes	Cytochrome P-450 content[a]	*N*-Oxide reductase activity[b]
Liver	28.5	27.2
Kidney	4.5	3.9
Lung	2.8	3.7
Intestine	ND[c]	0.9
Brain	ND	0.7
Heart	ND	0.6

[a] Expressed as nanomoles per amount of microsomal protein equivalent to 1.0 g of tissue (wet weight).

[b] Expressed as nanomoles per minute per amount of microsomal protein equivalent to 1.0 g of tissue (wet weight).

[c] ND, not detectable.

of microsomal NADPH-dependent *N*-oxide reductase activity. The liver microsomal fraction, which contained the highest amount of cytochrome P-450 per milligram of microsomal protein, was the most active in the reduction of tertiary amine *N*-oxides, whereas microsomes from tissues, such as intestine, brain, and heart in which cytochrome P-450 content was low or undetectable displayed very weak or negligible activity. The *N*-oxide reduction rate in microsomal fraction from these tissues was approximately proportionate to their content of cytochrome P-450.

The subcellular distribution pattern of tertiary amine *N*-oxide reductase in liver was compared with those of succinate dehydrogenase (mitochondrial marker enzyme) and glucose 6-phosphatase (microsomal marker enzyme) (Table 2). With imipramine *N*-oxide and tiaramide *N*-oxide as the respective substrates, 66% and 70% of the tertiary amine *N*-oxide reductase activities of nuclei-free homogenates were found in the microsomal fraction. A certain amount of *N*-oxide reductase activity was also detected in the well-washed mitochondrial fraction, which was almost free from microsomal contamination.[3] The reduction rate of *N,N*-dimethylaniline *N*-ox-

[3] M. Sugiura, K. Iwasaki, and R. Kato, manuscript in preparation.

TABLE 2

Subcellular distribution of NADPH-dependent N-oxide reductase activity in rat liver

Subcellular fractions were obtained from perfused rat liver and sonicated prior to assay. The numbers in parentheses indicate the percentage of total activity.

Fraction	NADPH-dependent *N*-oxide reductase			Succinate dehydrogenase[a]	Glucose 6-phosphatase
	Imipramine *N*-oxide	Tiaramide *N*-oxide	*N,N*-Dimethylaniline *N*-oxide		
	nmoles tertiary amine formed/mg/min			*units/mg/min*	*nmoles/mg/min*
Mitochondrial	0.32 (12%)	0.26 (11%)	2.51 (35%)	9.7 (78%)	40 (1%)
Lysosomal	0.43 (14%)	0.31 (11%)	2.16 (27%)	1.4 (10%)	112 (19%)
Microsomal	2.01 (66%)	1.89 (70%)	2.27 (30%)	0.2 (1%)	279 (80%)
Soluble	0.06 (9%)	0.05 (8%)	0.16 (9%)	0.3 (11%)	2 (0%)

[a] One unit is equivalent to an absorbance change of 1.0/10 min.

ide in the mitochondrial fraction was comparable to that observed in the microsomal fraction. The reductase activity found in the lysosomal fraction might have been due to microsomal contamination, since 20% of the glucose 6-phosphatase activity was recovered in the lysosomal fraction. Little *N*-oxide reductase activity was found in the soluble fraction. This result suggests that xanthine oxidase, which donates electrons to heteroaromatic amine *N*-oxides such as nicotinamide *N*-oxide and purine *N*-oxide (27, 28), did not donate electrons to imipramine *N*-oxide, tiaramide *N*-oxide, or *N,N*-dimethylaniline *N*-oxide. The characteristics and properties of *N*-oxide reductase in liver microsomes were then investigated.

Effect of protease digestion on microsomal NADPH-dependent N-oxide reductase activity. The effect of trypsin or subtilisin digestion of liver microsomes on NADPH-dependent *N*-oxide reductase activity and other microsomal constituents is illustrated in Table 3. Incubation of liver microsomes with 25 μg of trypsin or subtilisin per milligram of protein at 0° anaerobically for 15 hr solubilized NADPH–cytochrome *c* reductase activity by 95%. After digestion the level of cytochrome P-450 remained intact, and formation of P-420 was negligible. Probably, therefore, part of the decrease in NADPH-dependent *N*-oxide activity in the protease-digested fraction might have been due to destruction of the hydrophobic site of NADPH-cytochrome *c* reductase, leading to a deficiency in the interaction with cytochrome P-450. The residual NADPH-suppprted *N*-oxide reductase activity was completely recovered in the pellet fraction after centrifugation of the trypsin- or subtilisin-digested microsomes. NADPH-cytochrome *c* reductase and cytochrome b_5 cannot themselves reduce *N*-oxides, since protease-solubilized fractions containing considerable amounts of cytochrome *c* reductase and cytochrome b_5 showed little or no *N*-oxide reductase activity. The subtilisin-digested microsomal pellet, depleted of cytochrome b_5 by more than 90%, exhibited almost the same activity as the trypsin-solubilized microsomal pellet, indicating a lack of involvement of cytochrome b_5 as one of the obligatory components of the NADPH-supported *N*-oxide reductase enzyme system.

NADPH-dependent N-oxide reductase activity in liver microsomes. Imipramine *N*-oxide, tiaramide *N*-oxide, and *N,N*-dimethylaniline *N*-oxide were effective substrates for the liver microsomal enzyme system (Table 4). The reduction of these substrates was inhibited about 95% under an atmosphere of carbon monoxide. Michaelis-Menten kinetic analysis of the NADPH-supported reduction of imipramine *N*-oxide, tiaramide *N*-oxide, and *N,N*-dimethylaniline *N*-oxide gave estimated apparent K_m values of 0.22, 0.40, and 0.20 mM, respectively (Table 5).

Stoichiometry of rates of NADPH oxidation and tertiary amine N-oxide reduction in liver microsomes. To evaluate the mechanism of *N*-oxide reduction in liver micro-

TABLE 3

Effect of protease digestion on microsomal NADPH-dependent N-oxide reductase activity

Liver microsomes (10 mg of protein per milliliter) were incubated at 0° with 25 μg of trypsin or subtilisin per milligram of protein for 15 hr anaerobically in 0.05 M sodium phosphate buffer (pH 7.4) containing 25% glycerol. Digestion with trypsin was stopped by addition of soybean trypsin inhibitor (1 mg/mg of protein), and the digested microsomes were then centrifuged at 105,000 × *g* for 2 hr. The pellets obtained were suspended in 0.05 M sodium phosphate buffer (pH 7.4) and used for the enzyme assays. NADPH-dependent tertiary amine *N*-oxide reduction was measured using 1.0 mM imipramine *N*-oxide.

Treatment	NADPH-cytochrome *c* reductase	Cytochrome b_5 reductase	Cytochrome P-450 content	NADPH-dependent *N*-oxide reductase	Protein[a]
	nmoles/mg/min	*nmoles/mg/min*	*nmoles/mg*	*nmoles/mg/min*	%
Intact microsome	128	0.42	1.08	2.01	
Trypsin					
Digested	141	0.32	0.81	0.29	
Supernatant	659	0.32	0.00	0.00	40
Pellet	13	0.47	1.36	0.34	60
Subtilisin					
Digested	140	0.36	0.69	0.20	
Supernatant	463	0.81	0.00	0.00	32
Pellet	10	0.03	1.02	0.29	68

[a] Percentage of protein recovered in each fraction after protease digestion.

TABLE 4

NADPH-dependent N-oxide reductase activity in liver microsomes

Reaction mixtures, prepared as described in MATERIALS AND METHODS, were incubated at 37° for 10 min under an atmosphere of nitrogen or carbon monoxide. The concentration of protein in the reaction mixture was 5.0 mg/ml.

Substrate	*N*-Oxide reduction		Inhibition
	N_2	CO	
	nmoles/mg/min		%
Imipramine *N*-oxide	2.14	0.11	95
Tiaramide *N*-oxide	1.88	0.09	95
N,N-Dimethylaniline *N*-oxide	2.31	0.12	95

TABLE 5

Kinetic parameters of imipramine N-oxide, tiaramide N-oxide, and N,N-dimethylaniline N-oxide reduction in liver microsomes

K_m values for tertiary amine *N*-oxides and the V_{max} values were obtained from double-reciprocal plots of the reaction velocities against substrate concentrations in the presence of excess NADPH. Other experimental conditions were the same as described in MATERIALS AND METHODS.

Substrate	K_m	V_{max}
	mM	*nmoles/mg/min*
Imipramine *N*-oxide	0.22	2.63
Tiaramide *N*-oxide	0.40	2.50
N,N-Dimethylaniline *N*-oxide	0.20	3.23

somes, the NADPH oxidation rate was followed during the reduction of imipramine *N*-oxide under an atmosphere of nitrogen. In the absence of imipramine *N*-oxide a slight endogenous consumption of reduced pyridine nucleotide was observed during the first few minutes of the reaction; thereafter NADPH oxidation became markedly slower and almost completely disappeared. The increase in the rate of NADPH oxidation by the addition of imipramine *N*-oxide was linear for 10 min, and the amount of NADPH oxidized was nearly equal to the amount of imipramine formed (Table 6). These results indicated that 1 mole of NADPH was oxidized for each mole of imipramine formed from imipramine *N*-oxide. It is therefore reasonable to assume that tertiary amine *N*-oxide was converted stoichiometrically to tertiary amine and water, with the consumption of an equimolar amount of NADPH.

TABLE 6

Stoichiometry of rates of NADPH oxidation and tertiary amine N-oxide reduction in liver microsomes

The amount of NADPH oxidized during the stoichiometric study was measured under an atmosphere of nitrogen as the rate of decrease in optical density at 340 nm at 37°, using a Hitachi model 356 two-wavelength, double-beam spectrophotometer. The reaction was started by the addition of imipramine *N*-oxide; after 5 or 10 min the incubation was stopped by adding NaOH, and samples were removed for the measurement of imipramine formed and for the spectrophotometric measurement of NADPH oxidation. In this experiment Thunberg vessels and extra pure (99.999%) nitrogen gas were used. The increase in the rate of NADPH oxidation caused by the addition of imipramine *N*-oxide was linear for at least 10 min. Values are the means and standard errors of four determinations.

Expt.	NADPH	Imipramine *N*-oxide	NADPH oxidation rate in presence of imipramine *N*-oxide (A)	Imipramine formation rate (B)	A:B
	mM	*mM*	*nmoles/mg/min*	*nmoles/mg/min*	
1	1.00	0.75	2.15 ± 0.02	1.94 ± 0.08	1.10
2	0.75	1.00	1.61 ± 0.02	1.65 ± 0.07	0.98

Inhibition of NADPH-dependent N-oxide reduction by oxygen and carbon monoxide. The effects of oxygen and carbon monoxide concentrations on the reduction of imipramine *N*-oxide are shown in Fig. 2. The reduction was inhibited about 60% in the presence of 5 μM oxygen, and about 70% in the presence of 9 μM oxygen. It is well known that oxygen interacts with the ferrous heme moiety of cytochrome P-450 in the oxidative metabolism of drugs, steroids, and fatty acids. It is therefore quite possible that *N*-oxide binds to reduced cytochrome P-450 provided through NADPH and NADPH–cytochrome *c* reductase in a fashion analogous to oxygen binding in oxidative metabolism. The inhibition of the reduction of *N*-oxide caused by carbon monoxide was more intense than that observed with oxygen. Under a partial pressure of 0.5% carbon monoxide, the reduction of *N*-oxide was decreased about 70%. A 5% partial pressure of carbon monoxide almost completely inhibited NADPH-dependent *N*-oxide reduction. Double-reciprocal plots of various concentrations of imipramine *N*-oxide in the presence of 3.3 μM carbon monoxide gave an apparent K_i value for carbon monoxide of 0.55 μM. It is of considerable significance that the inhibition of *N*-oxide reductase activity under an atmosphere of 0.5% carbon monoxide was almost completely reversed by exposure to white light (Table 7). Furthermore, under an atmosphere of 5.0% carbon monoxide, a small but distinct increment in the reductase activity was observed. *N*-Oxide reduction did not take place during exposure to white light without NADPH or microsomes.

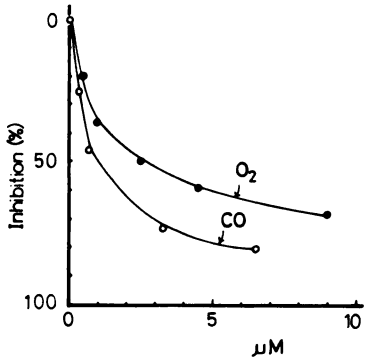


FIG. 2. *Effects of oxygen and carbon monoxide on NADPH-dependent N-oxide reductase activity*

NADPH-dependent *N*-oxide reductase activity from liver microsomes was assayed in the presence of 10 mM $MgCl_2$, 2 mM NADPH, 5 mg of microsomal protein per milliliter, 0.1 M phosphate buffer (pH 7.4), and 1.0 mM imipramine *N*-oxide under the indicated concentrations of oxygen (●) or carbon monoxide (○). Various O_2–N_2 and CO–N_2 mixtures were prepared in gas burettes and passed into Thunberg vessels containing the reaction mixture. The concentrations of carbon monoxide and oxygen in the aqueous phase were calculated from the partial pressure of carbon monoxide and oxygen and temperature. Simultaneous control reactions were carried out under an N_2 atmosphere.

Inhibition of NADPH-dependent N-oxide reductase activity by primary amines.

TABLE 7

Effect of exposure to white light on inhibition by carbon monoxide of tertiary amine N-oxide conversion to tertiary amines by liver microsomes

The reaction mixture (5 ml) contained 0.1 M phosphate buffer (pH 7.4), 2 mM NADPH, 10 mM $MgCl_2$, 25 mg of microsomal protein, and the indicated tertiary amine *N*-oxide at a concentration of 1.0 mM. Various CO-N_2 mixtures were prepared in gas burettes and passed into Thunberg vessels containing the reaction mixture. The incubations were carried out for 10 min at 37°. Numbers in parentheses indicate the percentage activity relative to values under an atmosphere of nitrogen in the dark.

Substrate	NADPH-dependent *N*-oxide reductase activity			Light reversal: (C − B)/(C − A) × 100
	With N_2, dark (A)	With CO, dark (B)	With CO, light[a] (C)	
	nmoles/mg/min			%
Experiment 1[b]				
Imipramine *N*-oxide	2.08	1.05 (50%)	1.87 (90%)	80
Tiaramide *N*-oxide	2.03	1.04 (51%)	1.90 (94%)	87
N,N-Dimethylaniline *N*-oxide	2.28	1.28 (56%)	2.10 (92%)	82
Experiment 2[c]				
Imipramine *N*-oxide	2.08	0.18 (9%)	0.47 (23%)	15
Tiaramide *N*-oxide	2.03	0.20 (9%)	0.54 (27%)	19
N,N-Dimethylaniline *N*-oxide	2.28	0.21 (7%)	0.76 (33%)	27

[a] Thunberg vessels in water were illuminated with a 500-W white lamp (Kokuyo) placed about 8 cm away.
[b] Under a gas phase of carbon monoxide (0.5%) and nitrogen (99.5%).
[c] Under a gas phase of carbon monoxide (5.0%) and nitrogen (95.0%).

It is generally accepted that the addition of type I compounds, such as SKF 525-A and imipramine, accelerates the reduction of cytochrome P-450 (29), while type II compounds, such as aniline, *n*-octylamine, and DPEA, which are assumed to coordinate directly to cytochrome P-450 in its heme region, retard the reduction of cytochrome P-450 (29–31). Therefore the inhibitory effects of aniline, *n*-octylamine, and DPEA on NADPH-dependent *N*-oxide reductase activity were investigated. As shown in Table 8, these primary amines inhibited the reduction of *N*-oxides, with DPEA inhibiting the reduction of imipramine *N*-oxide, tiaramide *N*-oxide, and *N,N*-dimethylaniline *N*-oxide by 51%, 92%, and 52%, respectively.

Another point of interest is that NADPH-dependent *N*-oxide reduction was markedly inhibited by other *N*-oxides. For example, imipramine *N*-oxide reduction was inhibited 59% and 24% by the addition of 1 mM *N,N*-dimethylaniline *N*-oxide and tiaramide *N*-oxide, respectively (Table 9). Tiaramide *N*-oxide reduction was inhibited 67% and 63% by the addition of 1 mM imipramine *N*-oxide and *N,N*-dimethylaniline *N*-oxide, respectively.

TABLE 8

Effects of type II ligands on NADPH-dependent N-oxide reductase activity in liver microsomes

NADPH-dependent *N*-oxide reductase activity was determined in the presence of the specified concentration of type II substances. The control activities for imipramine *N*-oxide, tiaramide *N*-oxide, and *N,N*-dimethylaniline *N*-oxide reductase were 2.01, 1.88, and 2.12 nmoles/mg/min, respectively.

Type II ligand	Inhibition		
	Imipramine *N*-oxide	Tiaramide *N*-oxide	*N,N*-Dimethylaniline *N*-oxide
	%	%	%
Aniline, 1 mM	29	40	28
n-Octylamine, 1 mM	42	84	53
DPEA, 0.1 mM	51	92	52

The addition of nitro and azo compounds to the incubation mixture also markedly reduced the reduction rate of *N*-oxides. This result is of interest because cytochrome P-450 in liver microsomes is also

TABLE 9

Effects of various compounds on NADPH-dependent N-oxide reductase activity in liver microsomes

NADPH-dependent *N*-oxide reductase activity was determined in the presence of 1 mM concentrations of the indicated compounds, using imipramine *N*-oxide as substrate. The control activity for imipramine *N*-oxide reduction was 2.01 nmoles/mg/min.

Compound	Inhibition
	%
N,N-Dimethylaniline *N*-oxide	58.7
Tiaramide *N*-oxide	24.4
p-Nitrobenzoic acid	62.7
α-Nitroso-β-naphthol	73.6
Nitrosobenzene	51.7
p-Dimethylaminoazobenzene	48.8
Cumene hydroperoxide	62.7
Benzylhydrazine	77.9

involved in the reduction of nitro and azo compounds (5). In addition, hydrazides and cumene hydroperoxide, which are also known to interact with cytochrome P-450 in its heme region (17), were found to be effective inhibitors of NADPH-dependent *N*-oxide reductase in liver microsomes.

Acceleration of NADPH-dependent N-oxide reductase activity by flavins. The addition of 150 μM FMN, FAD, or riboflavin to microsomes enhanced *N*-oxide reductase activity about 5-fold, and this effect was almost completely reversed by carbon monoxide. It is possible that the exogenously added flavins were transhydrogenated by NADPH and then accelerated the reduction of cytochrome P-450, followed by the enhancement of *N*-oxide reduction. During the preparation of this manuscript, Dajani *et al.* (32) reported a similar stimulation of nicotine *N*-oxide reduction by flavins. In connection with these observations, it is of considerable interest that the addition of reduced methyl viologen to microsomes caused rapid reduction of tertiary amine *N*-oxides to tertiary amines and that carbon monoxide inhibited *N*-oxide reduction by this system.[4]

Competition between carbon monoxide- and N-oxide-induced spectral changes. As reported for tiaramide *N*-oxide (13), imipramine *N*-oxide and *N,N*-dimethylaniline *N*-oxide did not produce any significant spectral change with oxidized cytochrome P-450. With reduced cytochrome P-450, on the other hand, tiaramide *N*-oxide produced spectral changes with a peak in the difference spectrum at 442 nm (13). As the magnitude of spectral change was higher in microsomes from phenobarbital treated rats, these microsomes were used throughout. The spectral changes produced by tertiary amine *N*-oxides depended on each substrate used, probably because of the unspecific or type I interaction of tertiary amines with cytochrome P-450.

To establish the binding site for tertiary amine *N*-oxides, competition between tiaramide *N*-oxide and carbon monoxide in the difference spectrum was studied, since the heme region of cytochrome P-450 may be the binding site for the *N*-oxide group. As shown in Fig. 3, the intensity of the ab-

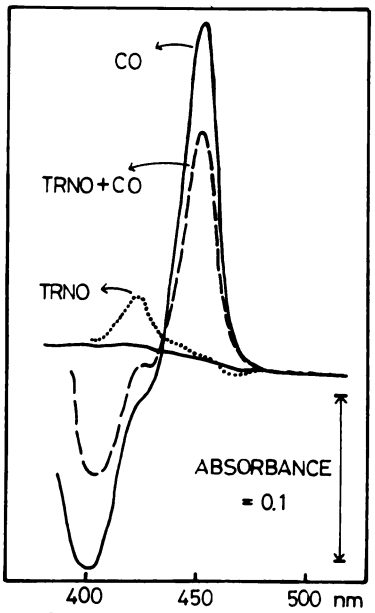


FIG. 3. *Competition between carbon monoxide and tertiary amine N-oxide for cytochrome P-450*

Liver microsomes (cytochrome P-450 content, 2.00 nmoles/mg of protein) from phenobarbital-treated rats, suspended in 0.2 M potassium phosphate buffer (pH 7.0), were used. The final concentration of microsomal protein was 3 mg/ml. Difference spectra were measured after treatment with dithionite and then added to the sample cuvettes as follows: ——, 0.005 volume of CO-saturated water; - - -, 5 mM (final concentration) of tiaramide *N*-oxide (TRNO); ····, both CO and tiaramide *N*-oxide.

[4] R. Kato, K. Iwasaki, and M. Sugiura, manuscript in preparation.

sorption at 450 nm produced by carbon monoxide was decreased by the addition of tiaramide *N*-oxide; likewise, the absorption at about 424 nm produced by tiaramide *N*-oxide was decreased by the addition of carbon monoxide. This evidence, combined with the marked inhibition of *N*-oxide reductase activity by *n*-octylamine and DPEA, which both form a hemichrome with reduced cytochrome P-450, supports the assumption that tertiary amine *N*-oxides combine with the reduced form of cytochrome P-450 and accept electrons directly from ferrous iron throughout the reaction.

Effect of phenobarbital or 3-methylcholanthrene treatment on microsomal NADPH-dependent N-oxide reductase activity. Phenobarbital or 3-methylcholanthrene treatment has been shown to increase hydroxylation of drugs, steroids, or polycyclic hydrocarbons and the specific content of cytochrome P-450 in liver microsomes without appreciably affecting the concentration of cytochrome b_5 (5). The effects of phenobarbital or 3-methylcholanthrene treatment on microsomal NADPH-dependent *N*-oxide reductase activity and other microsomal components are summarized in Table 10. The reduction of imipramine *N*-oxide and *N,N*-dimethylaniline *N*-oxide was increased 2.2- and 1.8-fold by prior treatment with phenobarbital. This activation was accompanied by a 1.8-fold increase in cytochrome P-450 content, while the amount of cytochrome b_5 in liver microsomes was not altered. Prior treatment of rats with 3-methylcholanthrene produced little or no enhancement of the reduction of imipramine *N*-oxide and *N,N*-dimethylaniline *N*-oxide, whereas the amount of cytochrome P-450 in liver microsomes was increased about 1.6-fold.

Influence of age and sex on NADPH-dependent N-oxide reductase activity in liver microsomes. Because cytochrome P-450-catalyzed drug oxidations, such as hexobarbital hydroxylation and aminopyrine *N*-demethylation, proceed faster in male than in female rat liver microsomes (33), it was of interest to examine *N*-oxide reductase activity in liver microsomes from rats of both sexes. As shown in Table 11, the sex difference is highly significant in the reduction of imipramine *N*-oxide and *N,N*-dimethylaniline *N*-oxide, but considerably less so in the reduction of tiaramide *N*-oxide.

It is generally accepted that the content of cytochrome P-450 in liver microsomes from newborn rats is extremely low but increases progressively with maturation (34, 35). The cytochrome P-450 content in these preparations varied widely, from 0.05 nmole/mg (newborn rats) to 1.02 nmoles/mg (mature male rats), as did the

TABLE 10

Effect of phenobarbital and 3-methylcholanthrene treatment on NADPH-dependent N-oxide reductase activity, NADPH-cytochrome c reductase activity, and cytochrome P-450 content of liver microsomes

Phenobarbital-treated rats were given the drug (90 mg/kg) intraperitoneally 72, 48, and 24 hr before death. 3-Methylcholanthrene-treated rats received injections of this compound (40 mg/kg) 48 and 24 hr before death. The respective control rats were treated either with NaCl or with olive oil. The results were obtained from three rats and are expressed as means ± standard errors.

Treatment	Imipramine *N*-oxide reduction	*N,N*-Dimethylaniline *N*-oxide reduction	NADPH-cytochrome *c* reductase	Cytochrome P-450
	nmoles/mg/min	*nmoles/mg/min*	*nmoles/mg/min*	*nmoles/mg*
NaCl	2.00 ± 0.12	2.76 ± 0.10	98 ± 7	1.02 ± 0.09
Phenobarbital (90 mg/kg, 3 days)	4.25 ± 0.31[a]	5.02 ± 0.88[a]	159 ± 11[a]	2.09 ± 0.20[a]
Olive oil	1.68 ± 0.25	2.41 ± 0.09	103 ± 8	1.00 ± 0.06
3-Methylcholanthrene (40 mg/kg, 2 days)	2.02 ± 0.15	2.57 ± 0.12	101 ± 8	1.56 ± 0.09[b]

[a] $p < 0.05$ compared with NaCl-treated controls.

[b] $p < 0.05$ compared with olive oil-treated control.

TABLE 11

NADPH-dependent N-oxide reductase activity in male and female rat liver microsomes

Reaction mixtures, prepared as described in MATERIALS AND METHODS, were incubated at 37° for 10 min under an atmosphere of nitrogen. The results were obtained from three rats and are expressed as means ± standard errors. The cytochrome P-450 concentrations in male and female rat liver microsomes were found to be 0.98 ± 0.03 and 0.77 ± 0.02 nmole/mg of protein, respectively.

Substrate	NADPH-dependent *N*-oxide reductase activity		A:B
	Male (A)	Female (B)	
	nmoles/mg/min		
Imipramine *N*-oxide	1.80 ± 0.08	0.84 ± 0.06	2.2
Tiaramide *N*-oxide	1.87 ± 0.06	1.46 ± 0.10	1.4
N,N-Dimethylaniline *N*-oxide	2.26 ± 0.08	1.03 ± 0.02	2.2

corresponding tiaramide *N*-oxide reductase activity, from 0.34 nmole/mg/min to 1.95 nmoles/mg/min.

Effects of various detergents on NADPH-dependent N-oxide reductase activity. Reagents which effectively convert cytochrome P-450 to cytochrome P-420 (e.g., detergents, protein-denaturing compounds, and sulfhydryl reagents) inactivate NADPH-dependent drug-metabolizing enzyme. Imai and Sato (36) reported that these reagents destroy the higher configuration of hydrophobic sites or interaction between phosphatidylcholine and cytochrome P-450 to cause the inactivation of NADPH-cytochrome P-450 reductase. NADPH-dependent *N*-oxide reductase activity was also abolished in deoxycholate-, potassium iodide-, or *p*-chloromercuribenzoate-treated microsomes, whereas considerable *N*-oxide reductase activity was recovered when reduced methyl viologen was used as an electron donor to replace the reduced pyridine nucleotide. *N*-Oxide reductase activity of these modified microsomes was heat-labile and sensitive to carbon monoxide. These results suggest that cytochrome P-420 in these modified microsomes is responsible for the observed *N*-oxide reductase activity of liver microsomes.[3]

DISCUSSION

We previously reported the possible involvement of cytochrome P-450 in the reduction of tiaramide *N*-oxide by rat liver microsomes (13). In the present work the characteristics and properties of *N*-oxide reductase activity were studied in detail to establish a common mechanism for the role of cytochrome P-450 in the reduction of tertiary amine *N*-oxides in liver microsomes. Of the various tissues examined, liver microsomes were the most active in converting tertiary amine *N*-oxides to their parent compounds. Low activities of *N*-oxide reduction were found with microsomes from kidney, lung, and intestine. Considerable activity, however, was found in the liver mitochondrial fraction, using *N,N*-dimethylaniline *N*-oxide as substrate. Bickel and collaborators (12, 37) could not demonstrate a clear localization of *N*-oxide reductase activity, using imipramine *N*-oxide and *N,N*-dimethylaniline *N*-oxide as substrates. The discrepancies between their work and ours may be due to differences in experimental conditions, such as supply of cofactor and anaerobicity. Reduction of nicotine *N*-oxide under a nitrogen atmosphere has also been recently reported (32).

The reduction of tertiary amine *N*-oxides in liver microsomes was sensitive to carbon monoxide and oxygen. In the presence of 1 μM carbon monoxide, NADPH-dependent *N*-oxide reductase activity was inhibited about 50%. The inhibition of *N*-oxide reductase activity under an atmosphere of 0.5% carbon monoxide was almost completely reversed by exposure to white light. These observations indicate that cytochrome P-450 is involved in the reduction of various tertiary amine *N*-oxide by liver microsomes.

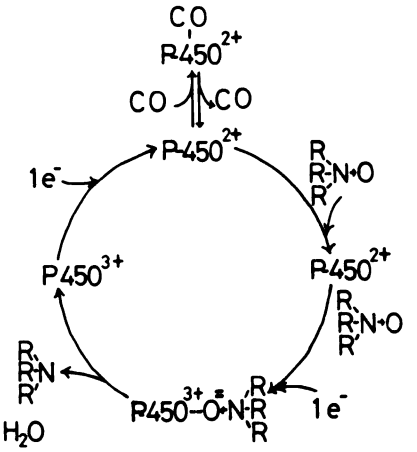
It has been reported that nitro and azo reductions are catalyzed by NADPH-cytochrome *c* reductase (8, 38) in addition to cytochrome P-450. In the present experiment, however, tertiary amine *N*-oxides were not reduced by either NADPH-cyto-

chrome *c* reductase or cytochrome b_5. Therefore tertiary amine *N*-oxides such as imipramine *N*-oxide and tiaramide *N*-oxide may serve as useful tools for the elucidation of cytochrome P-450-mediated reductive reactions.

It is generally accepted that cytochrome P-450 functions as an oxygen-activating component in hydroxylation reactions of drugs, steroids, and fatty acids under aerobic conditions and is able to react with ligand molecules such as oxygen, carbon monoxide, and cyanide at the sixth position of the heme moiety (2, 3). Now the question arises concerning the binding site for the *N*-oxide group on cytochrome P-450 under anaerobic conditions. Possible sites for substrate binding on cytochrome P-450 are a binding site for drugs or polycyclic hydrocarbons, a site to which primary amines bind to produce type II difference spectra, or the iron at the sixth position of the heme moiety of cytochrome P-450, where oxygen or carbon monoxide binds. Whether the binding site for primary amines is identical with the binding site for oxygen or carbon monoxide remains to be elucidated. As for the *N*-oxide binding site, the type I binding site can be excluded, since various drugs that produce type I difference spectra did not serve as effective inhibitors of *N*-oxide reduction; nor did imipramine *N*-oxide, tiaramide *N*-oxide, or *N,N*-dimethylaniline *N*-oxide produce any type I spectral changes. Therefore the likeliest binding site for the *N*-oxide group is the heme iron of cytochrome P-450. Several lines of available evidence favor this mode of binding. (a) *n*-Octylamine and DPEA, which bind to the heme iron of reduced cytochrome P-450 to form a hemichrome, exert a pronounced inhibition of NADPH-dependent *N*-oxide reduction. (b) Oxygen and carbon monoxide, which bind to the ferrous iron of heme as the sixth ligand, greatly inhibit NADPH-dependent *N*-oxide reduction. (c) Tiaramide *N*-oxide produced a spectral change with reduced cytochrome P-450 with a peak at 442 nm in the difference spectrum. The spectral changes produced by tertiary amine *N*-oxides, however, depend on the substrate used, probably because of the unspecific or type I interaction of tertiary amines with cytochrome P-450. (d) A considerable amount of imipramine was formed when imipramine *N*-oxide was incubated with EDTA and ferrous iron (10), whereas the formation of imipramine was negligible when ferric iron replaced ferrous iron.

We propose the following reaction mechanism for NADPH-dependent *N*-oxide reduction by cytochrome P-450: (a) reduction of cytochrome P-450 by NADPH, via NADPH–cytochrome *c* reductase; (b) binding of the *N*-oxide group to reduced cytochrome P-450 in its heme region; and (c) further reduction of the reduced cytochrome P-450–*N*-oxide complex, followed by reduction of the tertiary amine *N*-oxide to its corresponding tertiary amine and water (Scheme 1).

To summarize the characteristic nature of *N*-oxide reductase activity which involves cytochrome P-450 as a key participant, the properties of *N*-oxide reductase activity may be compared with those of oxidative metabolism (5, 17). First, the nature of the cytochrome P-450 that catalyzes *N*-oxide reductase activity should be distinguished from the oxidative activities of cytochrome P-450 regarding their sensitivities to oxygen and carbon monoxide. In oxidative metabolism catalyzed by cytochrome P-450, the intensity of inhibition



SCHEME 1. *Proposed mechanism of tertiary amine N-oxide reduction catalyzed by cytochrome P-450.*

depends on the ratio of carbon monoxide to oxygen (39), whereas in reductive metabolism, such as *N*-oxide reduction, the activity is inhibited by both carbon monoxide and oxygen. Second, oxidative reactions, such as steroid hydroxylation and drug oxidation, are considerably inhibited by type I ligands, including SKF 525-A. In contrast, type II reagents, such as DPEA, *n*-octylamine, and aniline, markedly inhibit tertiary amine *N*-oxide reductase activity (13, 40). Thus it is quite possible that during reactive states tertiary amine *N*-oxides coordinate directly with cytochrome P-450 in the heme region.

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