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PYRIDINE NUCLEOTIDE COFACTOR REQUIREMENTS OF INDICINE *N*-OXIDE REDUCTION BY HEPATIC MICROSOMAL CYTOCHROME P-450

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Abstract—Indicine *N*-oxide is reduced to indicine under anaerobic conditions by rat hepatic microsomal fraction in the presence of either NADH or NADPH. CO completely inhibits reduction, indicating the involvement of cytochrome P-450. In contrast to cytochrome P-450-catalyzed oxidations, for which NADH is about 15 per cent as effective as NADPH, NADH is 80 per cent as effective as NADPH in supporting indicine *N*-oxide reduction. In the presence of 3 mM NADH, the K_m for indicine *N*-oxide is 0.37 mM, and the V_{max} is 2.65 nmoles indicine formed/min/mg; with 3 mM NADPH the K_m is 0.51 mM, and the V_{max} is 3.00 nmoles/min/mg. NADH- and NADPH-dependent indicine *N*-oxide reduction appear to involve different pathways. NADH-supported reduction is inhibited 48 per cent by 0.5 mM KCN and 45 per cent by 0.8 M acetone, while NADPH-supported reduction is inhibited 3 per cent by 0.5 mM KCN and stimulated 28 per cent by 0.8 M acetone. The ability of NADH and NADPH at saturating concentrations to support indicine *N*-oxide reduction is additive, although this effect is not seen with phenobarbital- or 3-methylcholanthrene-pretreated animals. Phenobarbital pretreatment produces a selective increase in the V_{max} for NADPH-dependent reduction, to 5.75 nmoles/min/mg, but has no significant effect upon K_m with NADPH or upon either the K_m or the V_{max} for NADH-supported indicine *N*-oxide reduction. Pretreatment with 3-methylcholanthrene has no significant effect upon the K_m or V_{max} for NADPH- or NADH-supported reduction. A possible explanation for the observations is a form of cytochrome P-450 which can accept electrons from NADH and catalyze indicine *N*-oxide reduction but which does not contribute to oxidative microsomal drug metabolism.

The hepatic microsomal mixed-function oxidases exhibit a marked preference for NADPH as a source of reducing equivalents. NADH-supported microsomal oxidative drug demethylation takes place at 10–15 per cent of the rate seen with NADPH [1,2], and NADH is between 10 and 20 per cent as effective as NADPH in supporting the reduction of microsomal cytochrome P-450 under anaerobic conditions [1,3]. NADH is, however, more effective at supporting the reduction of tertiary amine *N*-oxides by the hepatic microsomal fraction. The maximum rates of reduction of imipramine *N*-oxide, tiaramide *N*-oxide and *N,N*-dimethylaniline *N*-oxide are about 50 per cent of those seen with NADPH [4,5]. Kato *et al.* [4,6] have shown that the microsomal reduction of tertiary amine *N*-oxides is mediated by cytochrome P-450. They proposed a reaction mechanism based upon the sequential two-electron reduction of cytochrome P-450, in a manner analogous to the reduction of cytochrome P-450 when it is acting as a mixed-function oxidase [4]. The rate-limiting step in the reduction of tertiary amine *N*-oxides has been suggested to be the reduction of cytochrome P-450 [7]. Such a mechanism does not account for the relative effectiveness of NADH in supporting cytochrome P-450-dependent drug reduction.

Indicine *N*-oxide is a pyrrolizidine alkaloid *N*-oxide currently undergoing clinical trial as an antitumor agent. It is reduced to indicine following intravenous administration in both experimental animals and man [8], although it is not yet clear whether indicine mediates the antitumor or toxic effects of indicine *N*-oxide [9]. In this paper, we report that NADH is almost as effective as NADPH in supporting microsomal cytochrome P-450-dependent reduction of indicine *N*-oxide. Some characteristics of the microsomal reduction of indicine *N*-oxide supported by NADH and NADPH are presented.

MATERIALS AND METHODS

Animals and preparation of the microsomal fraction. Male rats of the Sprague–Dawley strain (Sprague Dawley, Madison, WI), weighing between 150 and 200 g, were used. Animals were allowed free access to food and water at all times. Some animals were pretreated intraperitoneally with phenobarbital, 80 mg/kg daily, in saline for 3 days or with 3-methycholanthrene, 20 mg/kg daily, in corn oil (Mazola, CPC International, Englewood Cliffs, NJ) for 2 days. Preliminary studies indicated that corn oil had no effect upon microsomal indicine *N*-oxide reduction, and untreated control animals were used throughout the study. Livers were removed 24 hr after the last dose of phenobarbital or 3-methylcholanthrene and flushed retrogradely with 50 ml of cold 0.9% NaCl, the hepatic microsomal fraction was prepared by ultracentrifugation following homogenization in 0.25 M sucrose as described by Ernster *et al.* [10]. The microsomes were washed by resuspension in 20 vol. of 0.15 M KCl and then collected by ultracentrifugation before being suspended in 0.15 M KCl at a concentration of 10 mg protein/ml.

Protein was determined by the method of Lowry *et al.* [11], using crystalline bovine serum albumin as a standard.

Incubations and assays. The standard incubation (6 ml) contained 6 mg of microsomal protein, 0.6 mmole Tris–HCl buffer (pH 7.4), 30 μmoles $MgCl_2$, up to 18 μmoles indicine *N*-oxide, up to 18 μmoles NADH, and up to 18 μmoles NADPH. Indicine *N*-oxide is readily soluble in water. Incubations were conducted at 37° for 10 min with shaking under O_2, N_2 or CO, unless otherwise stated. A 10-min preincubation under the appropriate gas phase was employed, and the reaction was initiated by the addition of reduced pyridine nucleotide in 10 μl of degassed buffer. High purity N_2 or CO was passed through a deoxygenating solution described by Meites and Meites [12]. NADPH-cytochrome P-450 reductase was measured by a modification of the method of Gigon *et al.* [13] described previously [14]. Indicine formed from indicine *N*-oxide was detected by a gas chromatographic method described previously [15].

Drugs and chemicals. Indicine *N*-oxide was obtained from the Division of Cancer Treatment, National Cancer Institute, Bethesda MD, and was found to contain less than 0.33% indicine (by weight). NADPH and β-NADH were purchased from Boehringer Mannheim, Indianapolis, IN; crystalline bovine serum albumin, α-NADH, phenobarbital, hexobarbital and 3-methylcholanthrene were from the Sigma Chemical Co., St. Louis, MO.

Statistical treatment of results. Groups of data were subjected to Student's *t*-test [16] to determine if a significant difference existed between the means of the groups of data, at the 5 per cent level.

RESULTS

Indicine *N*-oxide was reduced to indicine by the hepatic microsomal fraction under anaerobic conditions with either NADPH or NADH as a source of reducing equivalents (Table 1). The rate of indicine formation was linear for at least 20 min (results not shown). β-NADH was more effective than α-NADH at supporting reduction with rates (± S.E. of mean, N = 3 microsomal preparations, $P < 0.05$) of 2.26 ± 0.07 nmoles/min/mg for β-NADH and 1.29 ± 0.23 nmoles/min/mg for α-NADH-supported reduction. Oxygen inhibited the reduction supported by either cofactor by 98 per cent, and carbon monoxide inhibited the reduction by at least 96 per cent (Table 1). Hexobarbital, a type I ligand, produced a 9 per cent inhibition of NADH-dependent reduction but had no significant effect when NADPH was the cofactor. β-Diethylaminoethyl diphenylpropylacetate (SKF 525-A), also a type I ligand, and an inhibitor of the microsomal mixed-function oxidase, had no significant effect upon NADH- or NADPH-dependent reduction. Aniline, a type II ligand, inhibited both NADH- and NADPH-dependent reduction by 23 and 28 per cent respectively. NADH-dependent reduction was more sensitive to the inhibitory effects of 0.5 mM KCN (48 per cent inhibition) than was the reduction supported by NADPH (3 per cent inhibition). Both NADPH- and NADH-dependent metabolism were inhibited almost 50 per cent by 5 mM KCN. Acetone (0.8 M) stimulated NADPH reduction of indicine *N*-oxide by 20 per cent but inhibited NADH-dependent reduction 45 per cent. Nitrite (1 mM) has been reported to be a potent inhibitor of microsomal azo reduction, although having no effect upon microsomal oxidative metabolism [17]. Indicine *N*-oxide reduction in the presence of NADPH and NADH was inhibited up to 81 per cent by 1 mM KNO_2.

The effects of varying the cofactor concentration on the reduction of indicine *N*-oxide are shown in Fig. 1. The V_{max} (± S.E.M., N = 4) for indicine formation observed with NADH was 81 per cent of that observed with NADPH as cofactor, 2.17 ± 0.15 and 2.69 ± 0.15 nmoles/min/mg respectively. The Lineweaver–Burk plot with NADH as cofactor showed an inflection at around 0.5 mM, suggesting that more than one process might be involved. This inflection was seen with four separate microsomal preparations but was not observed with NADPH as cofactor.

Table 1. Hepatic microsomal reduction of indicine *N*-oxide*

	Cofactor	
	NADPH (3 mM) (%)	NADH (3 mM) (%)
N_2	100.0 ± 5.2 (2.57)	100.0 ± 1.6 (2.08)
O_2	1.9 ± 0.3†	1.7 ± 0.5†
CO	2.1 ± 1.0†	4.2 ± 0.6†
N_2 + hexobarbital (5 mM)	109.9 ± 4.1	90.8 ± 1.5†
N_2 + SKF 525-A (0.5 mM)	104.4 ± 3.6	121.7 ± 16.9
N_2 + aniline (5 mM)	71.5 ± 7.7†	77.3 ± 8.3†
N_2 + KCN (0.5 mM)	96.6 ± 3.5	51.8 ± 2.9†
N_2 + KCN (5 mM)	50.9 ± 2.4†	51.7 ± 3.3†
N_2 + acetone (0.8 M)	128.5 ± 6.8†	55.2 ± 2.3†
N_2 + KNO_2 (1 mM)	18.4 ± 0.4†	22.7 ± 1.3†

* Metabolism was determined by the formation of indicine under anaerobic conditions over a 10-min period as described in the text. NADH and NADPH were present at an initial concentration of 3 mM. Values are means ± S.E.M. of four preparations, expressed as a precentage of the mean N_2 control value. Figures in parentheses are the control rates of anaerobic metabolism in nmoles/min/mg of microsomal protein.

† $P < 0.05$, compared to control value.

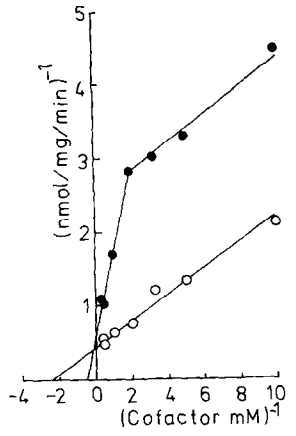

Fig. 1. Lineweaver–Burk plot of metabolism of indicine N-oxide (3 mM) with varying cofactor concentrations. Key: (○) NADPH; and (●) NADH.

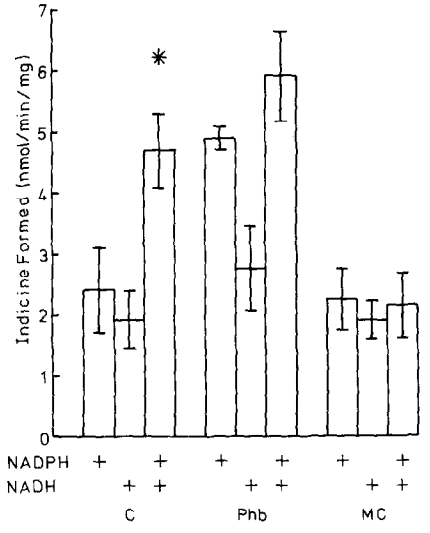

Fig. 2. Additive effects of NADPH and NADH in control and induced animals. Metabolism of indicine N-oxide was determined as described in the text with microsomes from C, control (3), Phb, phenobarbitone-induced (3) and MC, 3-methylcholanthrene-induced (4) rats; values in parentheses are numbers of animals. Bars are S.E. of mean. Substrate and individual cofactor concentrations are 3 mM. The asterisk (*) denotes $P < 0.01$, compared to NADPH alone.

The kinetic constants for the reduction of indicine N-oxide by the hepatic microsomal fraction from control and induced rats are shown in Table 2. There is no significant difference between the K_m value for indicine N-oxide with NADPH or NADH as the source of reducing equivalents in control, phenobarbital-pretreated or 3-methylcholanthrene-treated rats. Similarly, there is no significant difference between the V_{max} for indicine formation with NADPH or NADH in control or 3-methylcholanthrene-pretreated rats. Phenobarbital pretreatment led to a 92 per cent increase in the V_{max} for indicine formation with NADPH as the source of reducing equivalents, but had no significant effect upon the V_{max} with NADH as the source of reducing equivalents.

The abilities of NADPH and NADH at near saturating concentrations to support the reduction of indicine N-oxide were additive using microsomes from untreated rats (Fig. 2). Pretreatment of rats with either phenobarbital or 3-methylcholanthrene abolished the additive effect, and there was no significant difference between the rate of reduction with NADPH alone or with NADPH and NADH combined.

The effects of substrates upon the reduction of cytochromes P-450 by NADPH and NADH are

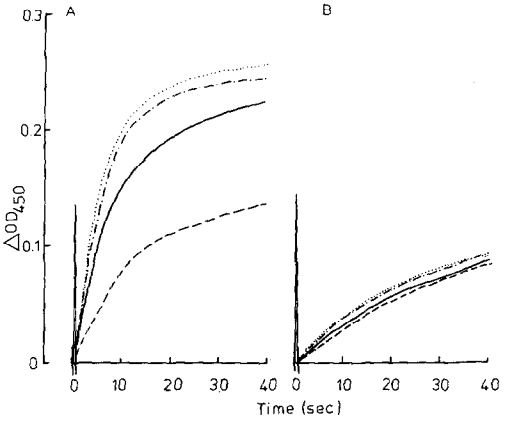

Fig. 3. Reduction of microsomal cytochtome P-450 by (A), NADPH and (B) NADH at 23°. Conditions: microsomal protein, 4 mg/ml; Tris–HCl buffer (pH 7.4), 0.15 M; $MgCl_2$, 5 mM; and NADPH or NADH at an initial concentration of 3 mM. Key: (—) control; (. . .) 5 mM aminopyrine; (- - -) 5 mM aniline; and (-·-·-) 5 mM indicine N-oxide.

Table 2. Effects of inducing agents on reduction of indicine N-oxide*

		NADPH		NADH	
Inducing agent	N	K_m (mM)	V_{max} (nmoles/min/mg)	K_m (mM)	V_{max} (nmoles/min/mg)
Control	4	0.51 ± 0.17	3.00 ± 0.68	0.37 ± 0.10	2.65 ± 0.59
Phenobarbital	3	0.67 ± 0.06	5.75 ± 0.61†	0.61 ± 0.17	2.98 ± 0.71
3-Methylcholanthrene	3	0.25 ± 0.03	2.71 ± 0.58	0.27 ± 0.07	2.17 ± 0.35

* Metabolism was determined as the formation of indicine under anaerobic conditions as described in the text. Pyridine nucleotide concentration was 3 mM. K_m relates to indicine N-oxide as the substrate, and V_{max} to the rate of indicine formation. Values are means ± S.E.M. of N different microsomal preparations, each from the pooled livers of three rats.

† $P < 0.05$, compared to control value.

shown as a typical recording in Fig. 3. The effects of the type I ligand aminopyrine and the type II ligand aniline, which facilitate and inhibit cytochrome P-450 reduction, respectively [13], are shown for comparison. Indicine *N*-oxide produced an increase in the rate of cytochrome P-450 reduction over the first 10 min with NADPH as a source of reducing equivalents (± S.E. of mean, N = 4 microsomal preparations), from 3.79 ± 0.41 to 4.78 ± 0.42 nmoles/min/mg ($P < 0.05$, paired *t*-test), and produced a small but nonsignificant increase in the reduction of cytochrome P-450 by NADH, from 0.43 ± 0.02 to 0.49 ± 0.03 nmoles/min/mg ($P > 0.05$, paired *t*-test).

DISCUSSION

Indicine *N*-oxide is reduced to indicine by rat liver microsomes by a process which requires reduced pyridine nucleotide as a source of reducing equivalents and which is inhibited by oxygen and carbon monoxide. Although inhibition by carbon monoxide is not conclusive evidence for the involvement of cytochrome P-450 [18], it is highly suggestive of cytochrome P-450-dependent metabolism, particularly when considering the hepatic microsomal fraction. Cytochrome P-450 has been demonstrated to be responsible for the anaerobic reduction of other tertiary amine *N*-oxides [4,7] and Iwasaki *et al.* [6] have shown that a reconstituted system containing cytochrome P-450 and NADPH-cytochrome P-450 reductase will reduce tiaramide *N*-oxide at a rate comparable to that with intact microsomes. The microsomal reduction of indicine *N*-oxide differs from the reduction of other tertiary amine *N*-oxides in not being stimulated by type I ligands [19], but is similar in its inhibition by aniline [4,5] and in being induced by phenobarbital but not by 3-methylcholanthrene [4,5].

Sugiura *et al.* [5] have reported that NADH can effectively support the anaerobic cytochrome P-450-dependent microsomal reduction of imipramine *N*-oxide, tiaramide *N*-oxide and *N, N*-dimethylaniline *N*-oxide at rates about 50 per cent of those seen with NADPH [4]. The present works shows that NADH is even more effective compared to NADPH at supporting the cytochrome P-450-dependent reduction of indicine *N*-oxide. The V_{max} for indicine formation with NADH as the cofactor is 2.6 nmoles/min/mg, and with NADPH it is 3.0 nmoles/min/mg.

The microsomal reduction of indicine *N*-oxide with NADH as the cofactor appears to differ in important respects from reduction with NADPH as the cofactor. Low concentrations of KCN (0.5 mM) inhibit NADH- but not NADPH-dependent reduction. The K_s of KCN for cytochrome P-450 is 2.5 mM [20], and microsomal mixed-function oxidase activity is not inhibited by 0.5 mM KCN [21]. Both NADH- and NADPH-dependent indicine *N*-oxide reduction are, however, inhibited by 5 mM KCN. Acetone stimulates NADPH-dependent reduction of indicine *N*-oxide but produces a marked inhibition of NADH-dependent metabolism. Acetone is an enhancer of the oxidative metabolism of certain type II ligands, such as aniline, and promotes NADH synergism of NADPH-dependent oxidative metabolism which is not normally seen with type II ligands [22]. Both NADPH- and NADH-supported indicine *N*-oxide reduction are inhibited by KNO_2, an inhibitor of microsomal azo reduction [17]. Azo compounds are reduced by cytochrome P-450 [23] but also, unlike indicine *N*-oxide, by NADPH-cytochrome P-450 reductase [24]. KNO_2 inhibition probably represents an interaction of NO_2^- with cytochrome P-450 under anaerobic conditions. Dimethylamino azobenzene has been reported to inhibit the microsomal reduction of imipramine *N*-oxide by both NADPH and NADH [4,5].

β-NADH is almost twice as effective as α-NADH in supporting the microsomal reduction of indicine *N*-oxide. This is suprising, since both forms of NADH have been reported to be equally effective at reducing cytochrome P-450 [25]. However, measuring cytochrome P-450 reduction by the formation of the reduced cytochrome P-450–CO complex probably only indicates the rate of introduction of the first electron to cytochrome P-450 and this may not be the rate-limiting step in tertiary amine *N*-oxide reduction.

Sugiura *et al.* [4] have proposed a mechanism for the reduction of tertiary amine *N*-oxides based upon the sequential two-electron reduction of cytochrome P-450 analogous to the reduction of cytochrome P-450 when it is acting as a mixed-function oxidase. Kato *et al.* [7] have further proposed that the rate-limiting step in the reduction of tertiary amine *N*-oxides is the reduction of cytochrome P-450. This suggested mechanism has to be reconciled with the findings that, with indicine *N*-oxide as a substrate, NADH supports metabolism at 80 per cent of the rate found with NADPH, while NADH reduces cytochrome P-450 at only 10–20 per cent of the rate seen with NADPH, both in the presence and absence of added substrate. The ability of NADH to support indicine *N*-oxide reduction is difficult to explain in terms of a slow rate of metabolism since the V_{max}, 2.6 nmoles/min/mg, is similar to that seen during the oxidative microsomal metabolism of, for example, norcodeine or aniline where NADH is relatively ineffective at supporting metabolism [26].

Immunochemical studies have suggested that NADPH-cytochrome P-450 reductase is an essential component in the electron flow from NADH to cytochrome P-450 for microsomal drug oxidation [27], while NADH-cytochrome b_5 reductase and cytochrome b_5 participate in the NADH-supported oxidation of some, but not of all drugs [26]. NADH-cytochrome b_5 reductase and cytochrome b_5 have been reported to mediate the flow of electrons from NADH to cytochrome P-450 for the reduction of tertiary amine *N*-oxides [5]. A possible explanation for the ability of NADH to support the reduction of indicine *N*-oxide and other tertiary amine *N*-oxides is that these compounds stimulate the transfer of electrons from reduced cytochrome b_5 to cytochrome P-450. The results of the present study show, however, that indicine *N*-oxide does not significantly increase the rate of reduction of microsomal cytochrome P-450 by NADH. Interpretation of the effects of indicine *N*-oxide upon cytochrome P-450 reduction, measured by the formation of the reduced cytochrome P-450–CO complex, is complicated by

the fact that CO and tertiary amine N-oxides bind competitively to the heme iron of cytochrome P-450 [4]. The presence of indicine N-oxide might thus affect the apparent rate of cytochrome P-450 reduction by displacing CO from reduced cytochrome P-450. The rate-limiting step in tertiary amine N-oxide reduction may not be the introduction of the first electron to cytochrome P-450, and it is possible that indicine N-oxide stimulates the transfer of the second electron to cytochrome P-450. It has been suggested that, in the presence of a substrate which enhances the flow of electrons to cytochrome P-450, the rate-limiting step in oxidative drug metabolism is the introduction of the second electron [28].

An alternative explanation for the ability of NADH to support cytochrome P-450-dependent indicine N-oxide reduction but not oxidative drug metabolism is that there is a form of cytochrome P-450 normally present in the microsomes which will readily accept electrons from NADH-cytochrome b_5 reductase or cytochrome b_5, but which will not function in the presence of oxygen or carbon monoxide. Miki *et al.* [29] have recently reported the purification from rabbit liver microsomes of a cytochrome P-450 with a high affinity for cytochrome b_5, which, in a reconstituted system with NADH-cytochrome b_5 reductase, cytochrome b_5, and detergent, is reduced by NADH. Evidence from the present study which lends support to the concept of two distinct pathways for the microsomal reduction of indicine N-oxide by NADH and NADPH is found in the different kinetic parameters, the different response to inhibitors and in the additive effects of NADH and NADPH at near saturating concentrations in supporting reduction. Phenobarbital or 3-methylcholanthrene pretreatment abolish the additive effects of NADH and NADPH, although phenobarbital selectively increases the maximal rate of reduction with NADPH as cofactor.

In summary, NADH has been found to be almost as effective as NADPH in supporting cytochrome P-450-mediated indicine N-oxide reduction. NADH-dependent metabolism differs in several respects from NADPH-dependent metabolism, particularly in the effects of inhibitors and of inducing agents. Possible reasons for the enhanced ability of NADPH to support reductive as opposed to oxidative metabolism are discussed, but the information currently available does not permit the unequivocal assignment of a mechanism to explain this difference.

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