STIMULATORY EFFECT OF FMN AND METHYL VIOLOGEN ON CYTOCHROME P-450 DEPENDENT REDUCTION OF TERTIARY AMINE N-OXIDE

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SUMMARY: FMN or methyl viologen stimulated anaerobic reduction of tertiary amine N-oxides by liver microsomes and this stimulatory effect was completely inhibited by carbon monoxide. Spectral study indicated that FMN or methyl viologen is reduced by NADPH-cytochrome c reductase and reduced FMN or methyl viologen is reoxidized by cytochrome P-450 in the presence of tertiary amine N-oxides. In the presence of FMN, xanthine oxidase-hypoxanthine system rapidly reduced tiaramide N-oxides through the reduction of cytochrome P-450: the maximum reduction rate of tiaramide N-oxide was about 100 nmoles/mg protein/min.

Introduction

In previous papers, we have reported that the reduction of tertiary amine N-oxides to corresponding tertiary amine in liver microsomes is catalyzed by the reduced form of cytochrome P-450 (1,2). This reduction is dependend on NADPH and NADH and is inhibited by both carbon monoxide and oxygen (2,3).

It has been found that the addition of FMN markedly stimulates the reduction rate of NADPH-dependent tertiary amine N-oxide by liver microsomes in anaerobic condition and the stimulated activity is almost completely inhibited by carbon monoxide (2).

In the present paper, the mechanism of stimulation of tertiary amine N-oxide reduction by FMN was investigated by comparison with the effect of methyl viologen on microsomal electron transport systems.

Materials and Methods

Male rats of Sprague-Dawley strain, age of 7 weeks, were used.

Liver microsomes were prepared as described in a previous paper (2). The microsomes were resuspended in 1.15 % KCl and recentrifuged to remove traces of contaminating hemoglobin.

The reductions of tertiary amine N-oxides were determined using a gaschromatography as described in the previous paper (2). The standard incubation mixture (2.5 ml) consisted of microsomal fraction (2.5 mg or 12.5 mg protein), substrate (2.5 μmoles), NADPH (5 μ moles), $MgCl_2$ (25 μmoles) and pH 7.4 phosphate buffer (150 μmoles).

NADPH, NADH, hypoxanthine, xanthine oxidase, FMN and methyl viologen were purchased from Sigma Chemical Co., St. Louis.

Results and Discussion

The addition of FMN or methyl viologen markedly stimulated the NADPH-dependent reductions of tiaramide N-oxide, imipramine-N-oxide and N,N-dimethylaniline N-oxide to corresponding tertiary amines as shown in Table 1. These stimulated activities were almost completely inhibited in an atmosphere of carbon monoxide.

Table 1. Effect of FMN or methyl viologen on NADPH-dependent reduction of tertiary amine N-oxide

Substrate	Gas phase	Tertiary amine N-oxide reductase (nmoles/mg/min)		
		Control	+FMN	+MV
Tiaramide N-oxide	N_2	2.13	15.16	17.10
	CO	0.20	0.11	0.39
Imipramine N-oxide	N_2	1.74	10.00	17.02
	CO	0.19	0.36	2.05
N,N-Dimethyl-aniline N-oxide	N_2	2.09	12.32	14.11
	CO	0.14	0.42	1.50

The concentrations of FMN and methyl viologen (MV) were 0.05 mM and 0.1 mM, respectively. Incubation was carried out under a strict anaerobic condition for 10 min. The protein concentration was 5 mg/ml. The activity of tertiary amine N-oxide reductase is expressed by the formation of the tertiary amine per mg microsomal protein per minute. The results are given as averages from two determinations.

Since the tertiary amine N-oxides were hardly reduced chemically by reduced forms of FMN and methyl viologen which are formed through addition of sodium dithionate under anaerobic condition, the involvement of cytochrome P-450 for the stimulatory mechanism of FMN or methyl viologen was suggested.

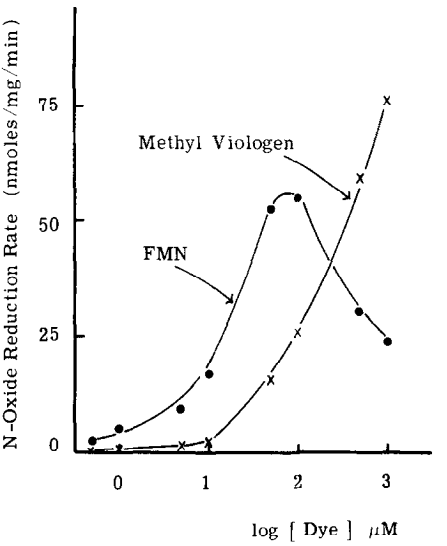

Fig. 1 Effect of various concentrations of FMN or methyl viologen on NADPH-dependent tiaramide N-oxide reduction. The amount of microsomal fraction was 2.5 mg protein. Incubation was carried out under a strict anaerobic condition for 10 min. The reduction rate is express as the amount of tiaramide formed per mg microsomal protein per minute. The control value was 0.70 nmole/mg/min. The results are given as averages from two determinations.

As shown in Fig. 1, the stimulatory effect of FMN on the reduction of tiaramide N-oxide was maximum at 10^{-4}M and significantly decreased at 10^{-3}M, whereas the stimulatory effect of methyl viologen was not saturated at 10^{-3}M. The maximum stimulation was 80-fold for FMN at 10^{-4}M and was 100-fold for methyl viologen at 10^{-3}M.

The NADH-dependent reductions of tiaramide N-oxide, imipramine N-oxide and N,N-dimethylaniline N-oxide to corresponding tertiary amines were also stimulated by FMN or methyl viologen (Table 2).

Table 2. Effect of FMN or methyl viologen on NADH-dependent reduction of tertiary amine N-oxide

Substrate	Gas phase	Tertiary amine N-oxide reductase (nmoles/mg/min)		
		Control	+FMN	+MV
Tiaramide N-oxide	N_2	0.94	9.91	3.89
	CO	0.04	0.06	0.06
Imipramine N-oxide	N_2	0.56	7.02	3.42
	CO	0.06	0.22	0.21
N,N-Dimethyl-aniline N-oxide	N_2	1.32	8.56	6.15
	CO	0.08	0.10	0.08

The concentrations of NADH, FMN and methyl viologen (MV) were 2.0 mM, 0.05 mM and 0.1 mM, respectively. Incubation was carried out under a strict anaerobic condition for 10 min. The protein concentration was 5 mg/ml. The results are given as averages from two determinations.

These stimulated activities were almost completely inhibited in an atmosphere of carbon monoxide.

These results suggest that FMN or methyl viologen is reduced through microsomal NADPH- and NADH-cytochrome c reductases and reduced form of FMN or methyl viologen then reduces cytochrome P-450 and the reduced form of cytochrome P-450 in its turn reduces the tertiary amine N-oxides.

To confirm this hypothesis, spectrophotometric studies on the reduction and oxidation of FMN by liver microsomes were carried out. As shown in Fig. 2, FMN was rapidly reduced by addition of NADPH. The maximum rate of FMN reduction was about 15 nmoles/mg protein/min and the initial rate of $FMNH_2$ oxidation induced by tiaramide N-oxide was about 33 nmoles/mg protein/min by spectrophotometric measurement.

In the presence of carbon monoxide the optical change of FMN was masked by the formation of cytochrome P-450-CO complex, but the

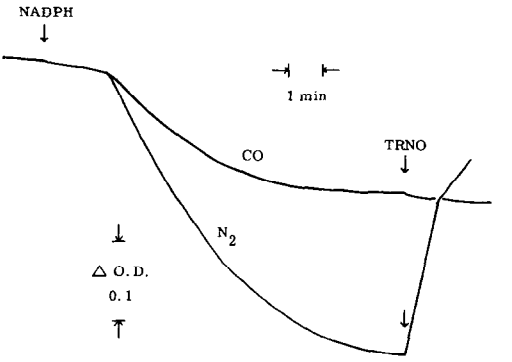

Fig. 2 NADPH- dependent reduction of FMN and its reoxidation by tiaramide N-oxide (TRNO) by liver microsomes. The reduction of FMN was measured by decrease in absorbance at 450 mμ in an atmosphere of nitrogen or carbon monoxide. The concentration of microsomal protein was 0.5 mg/ml. The volume of the incubation mixture was 2.5 ml. The concentrations of FMN and tiaramide N-oxide (TRNO) were 50 μM and 0.8 mM, respectively. The concentration of NADPH was 0.8 mM. One or two minute after the addition of tiaramide N-oxide the formation of tiaramide was determined by gaschromatography.

reoxidation of $FMNH_2$ induced by tiaramide N-oxide was clearly inhibited by carbon monoxide as observed in Fig. 2. The formation of tiaramide was 45.3 and 0.75 nmoles/mg protein/min, respectively under atmosphere of nitrogen and carbon monoxide. Similarly, spectrophotometric studies indicated that methyl viologen is rapidly reduced by liver microsomes on addition of NADPH and reoxidized on addition of tiaramide N-oxide. Carbon monoxide inhibited the oxidation of reduced methyl viologen.

A slow reduction rate of cytochrome P-450 by xanthine oxidase and hypoxanthine and its acceleration by FAD was reported by Ichikawa and Yamano (4). These results prompted us to use xanthine oxidase and hypoxanthine as artificial electron donor system for reduction of tertiary amine N-oxides.

In the absence of microsomes tiaramide N-oxide was not reduced directly by xanthine oxidase and hypoxanthine under atmosphere of nitrogen and the addition of xanthine oxidase and hypoxanthine to micro-

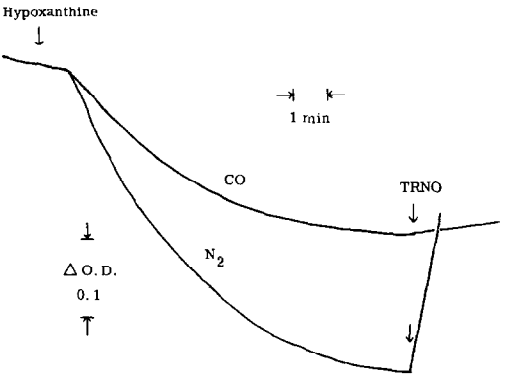

Fig. 3 Xanthine oxidase-dependent reduction of FMN and its re-oxidation by tiaramide (TRNO) by liver microsomes. NADPH was omitted, and the concentrations of xanthine oxidase and hypoxanthine were 0.4 U/ml and 4 mM, respectively. Other experimental conditions were the same as described in the legend in Fig. 2.

somes only slightly reduced tiaramide N-oxide under anaerobic atmosphere.

On the other hand, FMN was rapidly reduced by xanthine oxidase and hypoxanthine under anaerobic condition and the reduction of tertiary amine N-oxides was markedly stimulated (Fig. 3). The maximum rate of FMN reduction was about 18 nmoles/mg protein/min and the initial rate of $FMNH_2$ oxidation induced by tiaramide N-oxide was about 75 nmoles/mg protein/min by spectrophotometric determination. In the presence of carbon monoxide the optical change of $FMNH_2$ induced by tiaramide N-oxide was completely blocked.

The formation of tiaramide was 96.8 and 4.1 nmoles/mg protein/min, respectively under atmosphere of nitrogen and carbon monoxide.

Similarly, tiaramide N-oxide rapidly reoxidized the reduced form of methyl viologen reduced by xanthine oxidase and hypoxanthine and this reoxidation was inhibited by carbon monoxide.

In addition, xanthine oxidase-hypoxanthine system rapidly reduced tiaramide N-oxide in the presence of FMN and purified cytochrome P-450 (Kato, Iwasaki & Noguchi, manuscript in preparation).

These results support the view that FMN and methyl viologen

stimulate the reduction of tertiary amine N-oxide through the acceleration of reduction rate of cytochrome P-450. The reductions of FAD and methyl viologen by microsomal NADPH-cytochrome c reductase have been demonstrated (5,6).

In addition, Ichikawa and Yamano (4) have been reported that partially purified cytochrome P-450 is readily reduced by xanthine oxidase and hypoxanthine, and pigments such as methyl viologen, benzyl viologen, safranine T or FAD. However, hydroxylation of aniline and demethylation of aminopyrine and p-nitroanisole by these enzyme systems were not detected.

It has been postulated that the rate-limiting step of drug oxidation by liver microsomes is the step of cytochrome P-450 reduction by NADPH-cytochrome P-450 reductase (7,8). The addition of FAD or methyl viologen to microsomal drug-oxidation system significantly inhibited the oxidation of o-chloroaniline (9). This result may be due to ready oxidation of reduced form of FAD or methyl viologen by atmospheric oxygen, thus NADPH-dependent electron transport is diverted from cytochrome P-450 reduction pathway.

In the present study, it is demonstrated that the rate-limiting step in intact microsomes might be the step of reduction of cytochrome P-450. Thus in the presence of artifical electron donor system the reduction of tiaramide N-oxide is stimulated more than 100-fold.

The maximum turn-over rate of the reduction-oxidation, substrate binding and metabolite releasing circle of cytochrome P-450 in intact microsomes was calculated to be about 110 times per minute in the present experiment.

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