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INFLUENCE OF 2,4-DICHLORO-6-PHENOXYETHYLAMINE (DPEA) AND β-DIETHYLAMINOETHYL DIPHENYLPROPYLACETATE (SKF-525A) ON HEPATIC MICROSOMAL AZOREDUCTASE ACTIVITY FROM PHENOBARBITAL OR 3-METHYLCHOLANTHRENE INDUCED RATS*

LEON SHARGEL†‡ and PAUL MAZEL

Department of Pharmacology, The George Washington University, School of Medicine, Washington, D.C. 20005, U.S.A.

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Abstract—Studies with 2,4-dichloro-6-phenoxyethylamine (DPEA) and β-diethylaminoethyl diphenylpropylacetate (SKF-525A) demonstrated that the rate of reduction of an azo dye was altered by an interaction with cytochrome P-450 *in vitro*. Flavin-stimulated azoreductase activity was not affected by DPEA or SKF-525A. These compounds did not affect azoreductase activity associated with solubilized and partially purified NADPH cytochrome c reductase. Induction studies with phenobarbital and 3-methylcholanthrene indicated that both DPEA and SKF-525A acted at the carbon monoxide sensitive pathway which was induced by phenobarbital. DPEA inhibited azoreductase, whereas SKF-525A stimulated activity at the carbon monoxide sensitive site which is probably identical to cytochrome P-450.

A NUMBER of compounds which have little or no observable pharmacologic activity of their own prolong the action of other drugs when administered simultaneously. Two of these compounds, β-diethylaminoethyl diphenylpropylacetate (SKF-525A) and 2,4-dichloro-6-phenyl-phenoxyethylamine (DPEA) have been shown to be inhibitors of hepatic drug-metabolizing enzymes involved in the oxidation of drug substrates.[1–5]

The mechanism by which these compounds inhibit drug-metabolizing enzymes has not been fully elucidated. Anders and Mannering[2] have suggested that compounds such as SKF-525A act as alternate substrates for the microsomal enzymes. Furthermore, studies by Schenkman *et al.*[6] demonstrated that SKF-525A and DPEA interacted with microsomal cytochrome P-450 to produce spectral changes of the Type I and Type II modifications which were produced by other drug substrates. More recently, Gigon *et al.*[7] showed that SKF-525A stimulated, and DPEA inhibited, the reduction of hepatic microsomal cytochrome P-450.

The hepatic microsomal azoreductase system described by Hernandez *et al.*[8] and by

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† Predoctoral trainee supported by USPHS Training Grant 5 T1-GM26 from the National Institute of General Medical Sciences, NIH, Bethesda, Md.

‡ Present address: Sterling-Winthrop Research Institute, Rensselaer, N.Y. 12144.

Shargel and Mazel[9] is a composite of several enzymatic pathways: (1) an NADPH cytochrome c reductase pathway; (2) a cytochrome P-450 pathway; and (3) an NADPH specific pathway demonstrable by differential enzyme induction with phenobarbital (PB) or 3-methylcholanthrene (3MC). Thus, the hepatic azoreductase system can be useful in examining the effects of various drugs on microsomal electron transport enzymes.

The present study examines the effect of SKF-525A and DPEA on the microsomal reduction of the azo compound, neoprontosil. The results indicate that DPEA and SKF-525A can influence azoreductase activity by either inhibition or stimulation of the cytochrome P-450 pathway.

MATERIALS AND METHODS

Chemicals. Most reagents and solvents were purchased commercially. Neoprontosil was generously supplied by Sterling-Winthrop Research Institute, Rensselaer, N.Y.; DPEA was kindly donated by Dr. R. E. McMahon, Eli Lilly & Co., Indianapolis, Ind.; and SKF-525A was supplied by Smith, Kline & French Laboratories, Philadelphia, Pa.

Animals. Male, Sprague–Dawley rats, 100–125 g, maintained on Purina Laboratory Chow, were used for all studies. Induced animals were pretreated intraperitoneally with 3-methylcholanthrene, 40 mg/kg, dissolved in corn oil, or with phenobarbital, 90 mg/kg, dissolved in saline. Control animals received 0·5 ml of the vehicle. Animals were injected daily for 3 days and sacrificed by decapitation 24 hr after the last dose. Their livers were excised and immediately rinsed with ice-cold 1·15% KCl solution (in 0·01 M Na^+/K^+ phosphate buffer, pH 7·6, with 10^{-3} M EDTA). Microsomes were isolated as previously described.[10]

Enzymatic assays. Microsomal azoreductase activity was assayed by the method of Hernandez *et al.*[10] The following components in a final volume of 5 ml were added to serological flasks placed in an ice bath: 0·5 μmole NADP; 100 μmoles nicotinamide; 50 μmoles glucose 6-phosphate; soluble fraction from rat liver (equivalent to 250 mg wet weight); 10 μmoles neoprontosil; 5 μmoles EDTA; 0·05 M NaH_2PO_4–K_2HPO_4 buffer, pH 7·6; enzyme (equivalent to microsomes from 250 mg liver). While in the ice bath, the flasks were stoppered with serological stoppers and flushed with oxygen-free nitrogen for 5 min. The flasks were then incubated at 37° with shaking for 30 min in an atmosphere of pure nitrogen that was first passed through a deoxygenizer mixture (0·5% sodium dithionite, 0·05% 2-anthroquinone sodium sulfonate in 0·1 N sodium hydroxide). The reaction was terminated by addition of 15 ml of 6·67% trichloroacetic acid (TCA).

Sulfanilamide was separated from neoprontosil using a Dowex-50 column and determined by the method of Hernandez *et al.*[10] Previous experiments indicated that the concentration of nicotinamide used in the incubate did not affect azoreductase activity.

Microsomal protein was measured by the method of Lowry *et al.*[11] as modified by Miller[12] using crystalline bovine serum albumin as standard. NADPH cytochrome c reductase was solubilized from microsomes by treatment with 0·07% steapsin, and partially purified by precipitation in ammonium sulfate, 40–80 per cent saturation.[10]

RESULTS

Influence of SKF-525A and DPEA on the reduction of neoprontosil by hepatic microsomes

Effect of SKF-525A and DPEA on microsomal azoreductase in nitrogen or carbon monoxide atmospheres. The effect of SKF-525A and DPEA on microsomal azoreductase is shown in Table 1. SKF-525A, a tertiary amine, stimulated microsomal azoreductase activity under a nitrogen atmosphere, but had no effect on azoreductase under a carbon monoxide (CO) atmosphere. In contrast, DPEA, a primary amine, inhibited microsomal azoreductase activity in a nitrogen atmosphere, but did not affect enzyme activity under a CO atmosphere (Table 1).

In a recent study on nitroreductase, Sasame and Gillette[13] reported that this microsomal reductase activity was stimulated by SKF-525A and inhibited by DPEA. Thus, both azo and nitroreductase activities were affected similarly by these compounds.

TABLE 1. EFFECT OF SKF-525A AND DPEA ON THE REDUCTION OF NEOPRONTOSIL BY HEPATIC MICROSOMES*

	Drug	Concn(M)	Sulfanilamide formed (mμmoles/mg protein/30 min) Nitrogen atmosphere	Relative activity	Carbon monoxide atmosphere	Relative activity
I	Control		211 ± 10	100	152 ± 5	100
	SKF	10^{-3}	240 ± 10	114	148 ± 4	97
	SKF	10^{-4}	276 ± 8	131	156 ± 3	103
	SKF	10^{-5}	246 ± 16	117	151 ± 2	99
II	Control		197 ± 16	100	146 ± 11	100
	DPEA	10^{-3}	127 ± 12	64	121 ± 9	83
	DPEA	10^{-4}	158 ± 9	80	145 ± 12	99
	DPEA	10^{-5}	181 ± 13	92	141 ± 9	97

* The results are expressed as the mean ± S.E. of the values obtained from three individual experiments performed in duplicate from pooled rat liver microsomes. Inhibitors were preincubated with enzyme 20 min on ice prior to the addition of the reactants. Azoreductase activity was performed according to the procedure described in Materials and Methods.

Effect of SKF-525A and DPEA on flavin stimulation of microsomal azoreductase. The effect of DPEA and SKF-525A on FMN-stimulated azoreductase activity is presented in Table 2. DPEA, which inhibited microsomal azoreductase under nitrogen, did not inhibit enzyme activity stimulated by FMN. On the other hand, SKF-525A stimulated microsomal azoreductase under nitrogen, whereas the stimulation of azoreductase activity by FMN + SKF-525A was additive. Under a CO atmosphere, only the stimulatory effect of FMN was observed. The addition of DPEA + SKF-525A in equimolar concentrations had no effect on microsomal azoreductase activity either under nitrogen or CO. These results suggest that DPEA and SKF-525A act at a site on the microsomal enzymes which differs from the site in which flavin stimulates azoreductase activity. Furthermore, flavins such as FMN, FAD or riboflavin have been reported to stimulate azoreductase activity by stimulating microsomal NADPH cytochrome c reductase.[13,14]

TABLE 2. EFFECT OF SKF-525A AND DPEA ON FLAVIN STIMULATION OF MICROSOMAL AZOREDUCTASE*

Drug	Concn(M)	Sulfanilamide formed (mμmoles/mg protein/30 min) Nitrogen atmosphere	Relative activity	Carbon monoxide atmosphere	Relative activity
Control		191	100	157	100
Control + FMN	2×10^{-5}	262	137	215	137
SKF-525A	2×10^{-4}	266	139	150	96
SKF-525A + FMN	2×10^{-4}, 2×10^{-5}	335	175	208	133
DPEA	2×10^{-4}	165	86	155	99
DPEA + FMN	2×10^{-4}, 2×10^{-5}	236	123	215	137
DPEA + SKF-525A	2×10^{-4}, 2×10^{-4}	200	104	152	97

* Each value represents the mean of duplicate determinations on microsomes from ten pooled livers. Similar results were obtained in at least one other experiment. Inhibitors were preincubated with enzyme 20 min on ice prior to the addition of the reactants. Azoreductase activity was performed as described in Materials and Methods.

Effect of SKF-525A and DPEA on partially purified NADPH cytochrome c reductase enzyme

Hernandez *et al.*[10] have shown that steapsin solubilizes the microsomal enzyme, NADPH cytochrome c reductase, which was further purified by ammonium sulfate precipitation between 40–80 per cent saturation. This enzyme has azoreductase activity which is devoid of cytochrome P-450[10] but can be stimulated by flavins.[15]

The effect of DPEA and SKF-525A on the reduction of neoprontosil by this partially purified enzyme preparation (i.e. protein fraction that precipitates between 40–80% ammonium sulfate saturation) was investigated (Table 3). SKF-525A and DPEA did not affect the reduction of neoprontosil by the partially purified enzyme. FMN-stimulated azoreductase activity was unaffected by SKF-525A, whereas DPEA decreased the stimulation. However, at a concentration of 10^{-3} M, DPEA is poorly soluble and may have interfered with the stimulation of azoreductase by FMN. In addition, Cooper *et al.*[1] had reported that SKF-525A did not inhibit NADPH cytochrome c reductase activity in the intact microsomes.

Influence of SKF-525A and DPEA on microsomal azoreductase activity from phenobarbital and 3-methylcholanthrene pretreated rats

The results on Figs. 1 and 2 demonstrate that PB and 3MC pretreatment of rats induced azoreductase pathways that differ from each other in their sensitivity to CO inhibition and their sensitivity to DPEA and SKF-525A.

DPEA inhibited induced microsomal azoreductase activity in a similar manner to CO (Fig. 1). DPEA 10^{-4} M had no inhibitory effect on azoreductase activity under CO, whereas under a nitrogen atmosphere DPEA inhibited microsomal azoreductase activity of both controls and 3MC pretreated rats to the same extent (13 per cent). In

TABLE 3. EFFECT OF FMN AND INHIBITORS ON THE REDUCTION OF NEOPRONTOSIL BY THE PARTIALLY PURIFIED ENZYME*

Drug	Concn(M)	Sulfanilamide formed (mμmoles/mg protein/30 min)	
		Nitrogen atmosphere	Relative activity
Control		122	100
Control + FMN	2×10^{-5}	149	122
SKF-525A	10^{-3}	124	102
SKF-525A + FMN	10^{-3}, 2×10^{-5}	153	125
SKF-525A	10^{-5}	116	95
DPEA	10^{-3}	115	95
DPEA + FMN	10^{-3}, 2×10^{-5}	129	106
DPEA	10^{-4}	122	100
DPEA	10^{-5}	123	101

* Enzyme protein that precipitates between 40–80 per cent saturation ammonium sulfate. Each value represents the mean of duplicate determinations. Inhibitors were preincubated with enzyme 20 min on ice prior to the addition of the reactants. Azoreductase activity was performed as described in Materials and Methods.

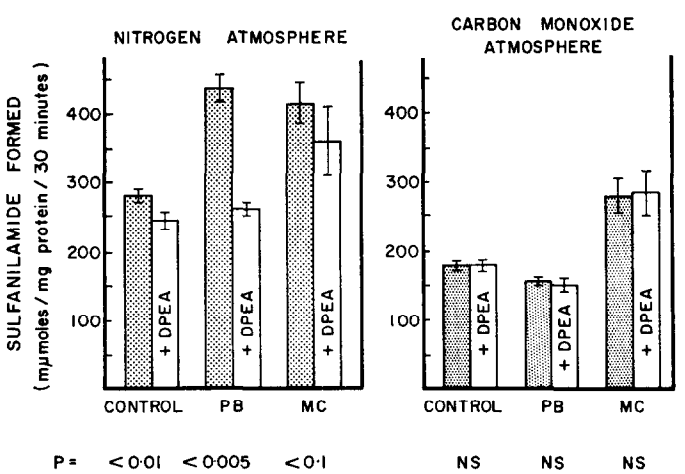

FIG. 1. Effect of DPEA on microsomal azoreductase from 3-methylcholanthrene and phenobarbital pretreated rats. Animal treatment is described in Materials and Methods. DPEA 10^{-4} M was preincubated with enzyme 20 min on ice prior to the addition of the reactants. The results are expressed as the mean ±S.E. of the values obtained from three separate groups of two pooled animals per group.

contrast, a significantly greater inhibition of azoreductase activity (40 per cent) by DPEA was observed in microsomes from phenobarbital pretreated rats.

The effect of SKF-525A on microsomal azoreductase activity from PB and 3MC pretreated rats was opposite to that of DPEA (Fig. 2). Under a nitrogen atmosphere, SKF-525A stimulated azoreductase activity (22 per cent) from microsomes of PB

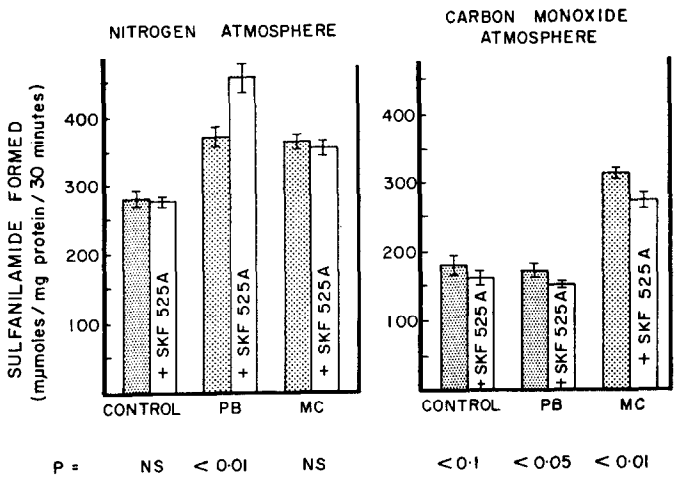

FIG. 2. Effect of SKF-525A on microsomal azoreductase from 3-methylcholanthrene and phenobarbital pretreated rats. Animal treatment is described in Materials and Methods. SKF-525A 10^{-4} M was preincubated with enzyme 20 min on ice prior to the addition of the reactants. The results are expressed as the mean ± S.E. of the values obtained from three separate groups of two pooled animals per group.

pretreated rats, but had very little effect on azoreductase activity from microsomes of control or 3MC pretreated animals (Fig. 2). Under CO, azoreductase activity of microsomes from all three groups was decreased by SKF-525A. The increased sensitivity of microsomal azoreductase activity from PB induced animals to be altered by SKF-525A or DPEA may be due to the fact that PB induces an azoreductase pathway that is more sensitive to CO inhibition, and is proportional to the increase in cytochrome P-450.[8]

DISCUSSION

The results demonstrate that the rate of reduction of neoprontosil by liver microsomal enzymes can be altered by an interaction with cytochrome P-450 *in vitro*. Gillette[16] and Gigon *et al.*[7] have postulated that the reduction of cytochrome P-450 might be the rate-limiting step in microsomal oxidations.

Under a nitrogen atmosphere, total azoreductase activity is measured, that is, the cytochrome P-450 component of azoreductase as well as the NADPH cytochrome c reductase component. Carbon monoxide blocks the cytochrome P-450 component of azoreductase activity. Therefore, incubations under an atmosphere of carbon monoxide yield azoreductase activity without the cytochrome P-450 pathway functioning.

DPEA and SKF-525A influenced the reduction of cytochrome P-450 since: (1) CO, which blocks cytochrome P-450, blocked the effect of DPEA and SKF-525A on microsomal azoreductase; (2) DPEA and SKF-525A do not block NADPH cytochrome c reductase; and (3) DPEA and SKF-525A had no effect on azoreductase activity of the partially purified enzyme which is identical to NADPH cytochrome c reductase. Moreover, our findings that SKF-525A stimulated microsomal azoreductase activity and DPEA depressed activity correlate with the findings of Gigon *et al.*[7] that SKF-525A increased cytochrome P-450 reductase activity, whereas DPEA depressed

cytochrome P-450 reductase activity. The actions of DPEA and SKF-525A on microsomal azoreductase agree with the findings of Sasame and Gillette[13] who found that drugs having a Type II spectral interaction with cytochrome P-450 inhibit nitroreductase by slowing the rate of cytochrome P-450 reduction.

Flavins affected microsomal azoreductase activity at a site on the electron transport chain that differed from the site for DPEA or SKF-525A. Stimulation of microsomal azoreductase activity by both SKF-525A and FMN *in vitro* was greater than the stimulation by each compound alone. DPEA depressed azoreductase activity, but did not prevent FMN-stimulated activity. The fact that CO inhibited the influence of both DPEA and SKF-525A on microsomal azoreductase indicated that these compounds acted at the cytochrome P-450 site.

The effect of DPEA and SKF-525A on microsomal azoreductase activity of PB or 3MC pretreated rats demonstrated differences in the induced microsomal enzyme pathways (Figs. 1 and 2). Microsomal azoreductase activity induced by PB was inhibited by DPEA to a greater extent than was the activity from control and 3MC pretreated rats. Takemori and Mannering[17] found that SKF-525A inhibited morphine demethylase which was not inducible by 3MC. SKF-525A did not affect the demethylation of 3-methyl-4-monomethyl-aminoazobenzene, which was inducible by 3MC. Our findings indicate that DPEA inhibited and SKF-525A stimulated the CO-sensitive pathway of microsomal azoreductase induced by PB. These inhibitors had little effect on the CO-insensitive microsomal azoreductase activity induced by 3MC.

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