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Effect of *p*-amino-diphenyl ethers on hepatic microsomal cytochrome P450

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The present paper aims to investigate whether *p*-amino-2′,4′-dichlorodiphenyl ether and *p*-amino-4′-methyldiphenyl ether are inhibitors as well as inducers of P450. Mice were given daily intraperitoneal (ip) injections of p-amino-2′,4′-dichlorodiphenyl ether (0.25 mmol/kg) or *p*-amino-4′-methyldiphenyl ether (0.25 mmol/kg) for 4 days and tested at 24 h and 48 h after the last dose injection. The results showed the mice pentobarbital sleeping time was shorter and the P450 content of hepatic microsome increased significantly in the group pretreated with *p*-amino-4′-methyldiphenyl ether when compared with the control group, while in mice pretreated with *p*-amino-2′,4′-dichlorodiphenyl ether the hepatic microsome P450 content increased but the pentobarbital sleeping time was extended in clear contrast to the control group. The sleeping time of the phenobarbital group (80 mg/kg daily ip injection for 4 days) was shortened at 24 h after the last injection with increased P450 content of hepatic microsome, but it showed no difference at 48 h. The zoxazolamine-paralysis times of mice treated with *p*-amino-2′,4′-dichlorodiphenyl ether were longer than those of the control mice, while the same dose of zoxazolamine did not lead to paralysis in mice pretreated with BNF. *p*-amino-2′,4′-dichlorodiphenyl ether and *p*-amino-4′-methyldiphenyl ether inhibited the activity of 7-ethoxyresorufin *O*-deethylase from rat hepatic microsome induced by BNF *in vitro* by 70.0% and 50.1% respectively. These results suggest that *p*-amino-2′,4′-dichlorodiphenyl ether and *p*-amino-4′-methyldiphenyl ether are inhibitors as well as inducers of P450.

1. Introduction

Cytochrome P450, primary member of hepatic mixed function oxidase (MFO) system can be inhibited or induced by various chemicals. A mechanism of induction has been suggested in which the active site of P450 is occupied by the inducer [1]. This deprives the body of P450 activity, so that the P450 gene is no longer inhibited, and as a result, the synthesis of P450 protein increases. In this sense, the inhibitor is the inducer. Studies have shown that animals given P450 inhibitor for a long time, P450 was induced. SKF-525A and ethanol are typical compounds with the dual actions of inhibition and reduction [2]. Substituted *p*-amino-diphenyl ethers (ADEs) are quasi-irreversible inhibitors of P450 that can form metabolic intermediate (MI) complex with P450 *in vitro* [3]. The mice pentobarbital sleeping time is a standard indirect measure of P450 activity [4]. In our previous study, the mice sleeping time was prolonged at 4 hours after intraperitoneal (ip) injection of ADEs, which suggested that ADEs are inhibitors of P450[5]. Among the seventeen substituted ADEs we have studied, *p*-amino-2′,4′-dichlorodiphenyl ether (diCl-ADE) is the strongest inhibitor of P450, white *p*-amino-4′-methyldiphenyl ether (Me-ADE) is a moderate inhibitor. This study was designed to find out whether these two substituted ADEs are inducers as well as inhibitors of P450.

2. Investigations and results

As shown in Table 1, the mouse pentobarbital sleeping time of the group pretreated with *p*-amino-4′-methyldiphenyl ether (Me-ADE) was shorter than that of the control group, although the P450 content of hepatic microsome increased significantly (Table 2). While the hepatic microsomal P450 content increased in mice pretreated with *p*-amino-2′,4′-dichlorodiphenyl ether (diCl-ADE), the pentobarbital sleeping time was extended in obvious contrast to the control group. The sleeping time of the phenobarbital group was shortened at 24 h after the last injection (shown in Table 1) with an increase in P450 content of hepatic microsome (Table 2), but it showed no difference at 48 h (Table 2).

Table 1: Influence of *p*-amino-diphenyl ethers (ADEs) on pentobarbital sleeping time (min) at 24 h and 48 h after the last injection of ADEs

Treatment	n	24 h	48 h
Control	12	32.3 ± 11.3	30.8 ± 10.9
diCl-ADE	12	35.1 ± 13.1	40.9 ± 5.7*
Me-ADE	12	19.0 ± 7.3*	17.8 ± 4.3*
Phenobarbital	12	7.3 ± 3.1**	24.8 ± 9.9

compared with the control group, * $P < 0.05$, ** $P < 0.01$

Table 2: Influence of p-amino-diphenyl ethers (ADEs) on microsome P450 content in hepatic microsomes of mice and hepatic weight

Treatment	n	P450 content# (n mol/g hepatic weight)	hepatic weight (g)
Control	12	3.0 ± 0.8	3.3 ± 1.7
diCl-ADE	12	5.2 ± 2.0*	3.6 ± 0.6
Me-ADE	12	5.1 ± 1.4*	3.9 ± 0.6
Phenobarbital	12	10.6 ± 2.5**	4.3 ± 0.6*

Three livers were combined for preparation of the microsomes, resulting in 4 samples; compared with control group, * $P < 0.05$, ** $P < 0.01$

Table 3: Mouse zaxazolamine paralysis time at 24 h after last ip injection of p-amino-diphenyl ethers (ADEs) and inhibitory rate of 7-ethoxyresorufin O-deethylase of BNF induced rat hepatic microsome by ADEs *in vitro*

T+reatment	Paralysis time (min)#	Inhibitory percentage (%)
Control	33.2 ± 16.5	0
diCl-ADE	73.8 ± 19.7*	70.0
Me-ADE	N.T.	50.1
BNF	0	N.T.

n = 12; * compared with control group, $P < 0.01$; N.T. means not tested

Table 3 indicates that in mice pretreated with diCl-ADE, the zaxazolamine paralysis time was prolonged significantly in contrast to the time for the control group. However, if the mice were pretreated with BNF, the same dose of zoxazolamine could not cause paralysis.
The inhibition was also confirmed *in vitro* by the finding that diCl-ADE and Me-ADE inhibit the activity of 7-ethoxyresorufin O-deethylase of hepatic microsome induced by BNF (Table 3).

3. Discussion

Pentobarbital is a central inhibitor, metabolized by cytochrome P450 in the hepatic microsomes. Therefore, the variation of pentobarbital sleeping time might be accounted for by changes in the activity of P450. Our study showed that *p*-amino-4′-methyldiphenyl ether (Me-ADE) is an inducer and inhibitor of P450, because it reduced the pentobarbital sleeping time and increased the P450 content of hepatic microsome. Contrary to our expectation, the result showed that the mice given *p*-amino-2′,4′-dichlorodiphenyl ether (diCl-ADE) exhibited both increased hepatic microsome P450 content and extended pentobarbital sleeping time. These results can be explained by the fact that diCl-ADE forms a stable MI complex in vivo, so that although it induces P450, the P450 was inactive because of forming an MI complex. During the preparation of the hepatic microsome *in vitro*, P450 was decomposed from the P450-MI complex, so the measured P450 content increased. Therefore diCl-ADE was an inhibitor as well as an inducer of P450. Me-ADE can also form an MI complex *in vivo*, but possibly it is unstable, so we can observe an increase in both sleeping time and P450 content. Phenobarbital is a well known inducer of P450, which can induce synthesis of P450, but cannot form an MI complex, so the sleeping time was extended significantly only at 24 h after last injection.

Zoxazolamine is a muscle paralyzing agent which is metabolized by hepatic microsome P4501A. The zoxazolamine paralysis time can reflect the activity of P4501A. Our result indicated that in mice pretreated with *p*-amino-2′,4′-dichlorodiphenyl ether (diCl-ADE), the zaxazolamine paralysis time was prolonged significantly in comparison with the control group. However, if the mice were pretreated with BNF, a inducer of P4501A, the same dose of zoxazolamine did not cause paralysis. The result suggests that diCl-ADE inhibits the activity of P4501A *in vivo*. 7-Ethoxyresorufin is a specific substrate of P4501A, and 7-ethoxyresorufin *O*-deethylase can be induced by BNF. The study showed that diCl-ADE, and *p*-amino-4′-methyldiphenyl ether (Me-ADE) inhibit the activity of 7-ethoxyresorufin *O*-deethylase of hepatic microsome induced by BNF. It means that the two ADEs are inhibitors of P4501A *in vitro*. In addition, a P4501A MI complex with hepatic microsome induced by BNF was not found, but the substrate-P4501A complex was observed *in vitro* (data not shown). However the two ADEs can form MI complexes with P450. We conclude that the inhibitory mechanism of diCl-ADE and of Me-ADE on P4501A are different from the mechanism with other P450s.

4. Experimental

4.1. Chemicals and animals

NADPH, BNF and zoxazolamine were obtained from Sigma Chemical Co, and *p*-amino-2′,4′-dichlorodiphenyl ether (diCl-ADE) and *p*-amino-4′-methyldiphenyl ether (Me-ADE) were synthesized at the Institute of Element-Organic Chemistry, Nankai University. All other chemical were AR reagents.
Male Sprague-Dawley rats (180–220 g) and NIH mice (20–25 g) were obtained from the Experimental Animal Centre of the Medical College, Zhejiang University.

4.2. Pentobarbital sleeping time

The effect of the two *p*-amino-diphenyl ethers on cytochrome P450 activity was measured in mice indirectly by measuring the pentobarbital sleeping time. For 4 days, mice were given daily intraperitoneal (ip) injections of *p*-amino-2′,4′-dichlorodiphenyl ether (12 mice, 0.25 mmol/kg) or *p*-amino-4′-methyl-diphenyl ether (12 mice, 0.25 mmol/kg daily). Another group of 12 mice served as a positive-control group and received daily ip injections of phenobarbital (80 mg/kg daily), a well known inducer of P450. A fourth group of 12 mice served as the control group and received daily ip injections of saline. At 24 h and 48 h after the last injection, each mouse was given pentobarbital (50 mg/kg) in the tail vein. The sleeping time of each animal after receiving the pentobarbital was determined by observation.

4.3. Measurement of P450 content

Mice were pretreated as described above for 4 days. The animals were fasted overnight and decapitated at 24 h after the last injection. Each liver was weighted and hepatic microsomes were prepared according to the procedure described by Franklin and Estabrook [6], and then the P450 contents of the microsomes were measured according to Omura and Sato [7].

4.4. Zoxazolamine paralysis time

The effects of *p*-amino-2′,4′-dichlorodiphenyl ether (diCl-ADE) on cytochrome P4501A activity *in vivo* were measured indirectly in mice by measuring the zoxazolamine paralysis time. The experimental groups and control group were pretreated as described in 4.2., while the positive-control group was given (ip) injections of BNF (80 mg/kg daily), a well known inducer of P4501A. At 24 h after the last injection, zoxazolamine (100 mg/kg) was given (ip). The time each mice remained paralyzed was recorded.

4.5. Determination of inhibitory rate of 7-ethoxyresorufin O-deethylase

Male Sprague-Dawley rats (180–220 g) were injected (i.p) with BNF (80 mg/kg) daily for 3 days. The hepatic microsomes were prepared, the

protein concentration was measured according to Lowry et al. [8], and the percentage inhibition of 7-ethoxyresorufin *O*-deethylase was determined by the method of Alan [9]. The studies were performed with microsomes suspended in phosphate buffer solution (0.1 mol/L, pH 7.4) at 200 μg/ml of microsomal protein, 1.7 μmol/L of 7-ethoxyresorufin and 25 μmol/L of diCl-ADE or Me-ADE.

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