Influence of Aroclor 1254, phenobarbital, β-naphthoflavone, and ethanol pretreatment on the biotransformation of cyclophosphamide in male and female rats

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Abstract

The aim of the present study is to investigate the influence of the environmental factors, smoking and alcohol, on the biotransformation of cyclophosphamide (CP) in the rat in vivo and in vitro with S9 liver fractions. The biotransformation of CP was studied by the determination of the CP metabolites, nor-nitrogen mustard (NNM), 4-ketocyclophosphamide (KCP), and carboxyphosphamide (CAR). The effect of the environmental factors, smoking and alcohol consumption, on the biotransformation enzymes was mimicked by pretreatment of rats with β-naphthoflavone and ethanol, respectively. Rats treated with olive oil and water served as controls and rats pretreated with Aroclor 1254 and phenobarbital were used as positive controls. The influence of sex and supplementation with NAD and GSH, mimicking a biological variation in NAD and GSH levels in rat and human liver, was also studied. Pretreatment of rats with Aroclor 1254 decreased the excretion of unmetabolized CP in urine, most likely due to an enhanced biotransformation. The in vitro hepatic biotransformation of CP in rats was strongly influenced by sex, by supplementation with NAD and GSH, and by pretreatment with the enzyme-inducers, phenobarbital and Aroclor 1254. No influence of pretreatment with the enzyme-inducers, β-naphthoflavone and ethanol, was found. The results suggest that the influence of the environmental factors, alcohol consumption and smoking, on the biotransformation of CP in man will be negligible.

Keywords: Cyclophosphamide; Biotransformation; Rat; Cytochrome P450; Liver

1. Introduction

Cyclophosphamide (CP) is an important anti-neoplastic drug used in the chemotherapy of cancer and autoimmune diseases. The activity of CP is caused by its metabolites (Sladek, 1988). During bioactivation by hepatic cytochrome P450, 4-hydroxycyclophosphamide is formed which is in equilibrium with its ring-opened tautomer aldophosphamide (Fig. 1). Both metabolites are detoxified by isoenzymes of the NAD-linked aldehyde oxidase and aldehyde dehydrogenase resulting in the formation of 4-ketocyclophosphamide (KCP) and carboxyphosphamide
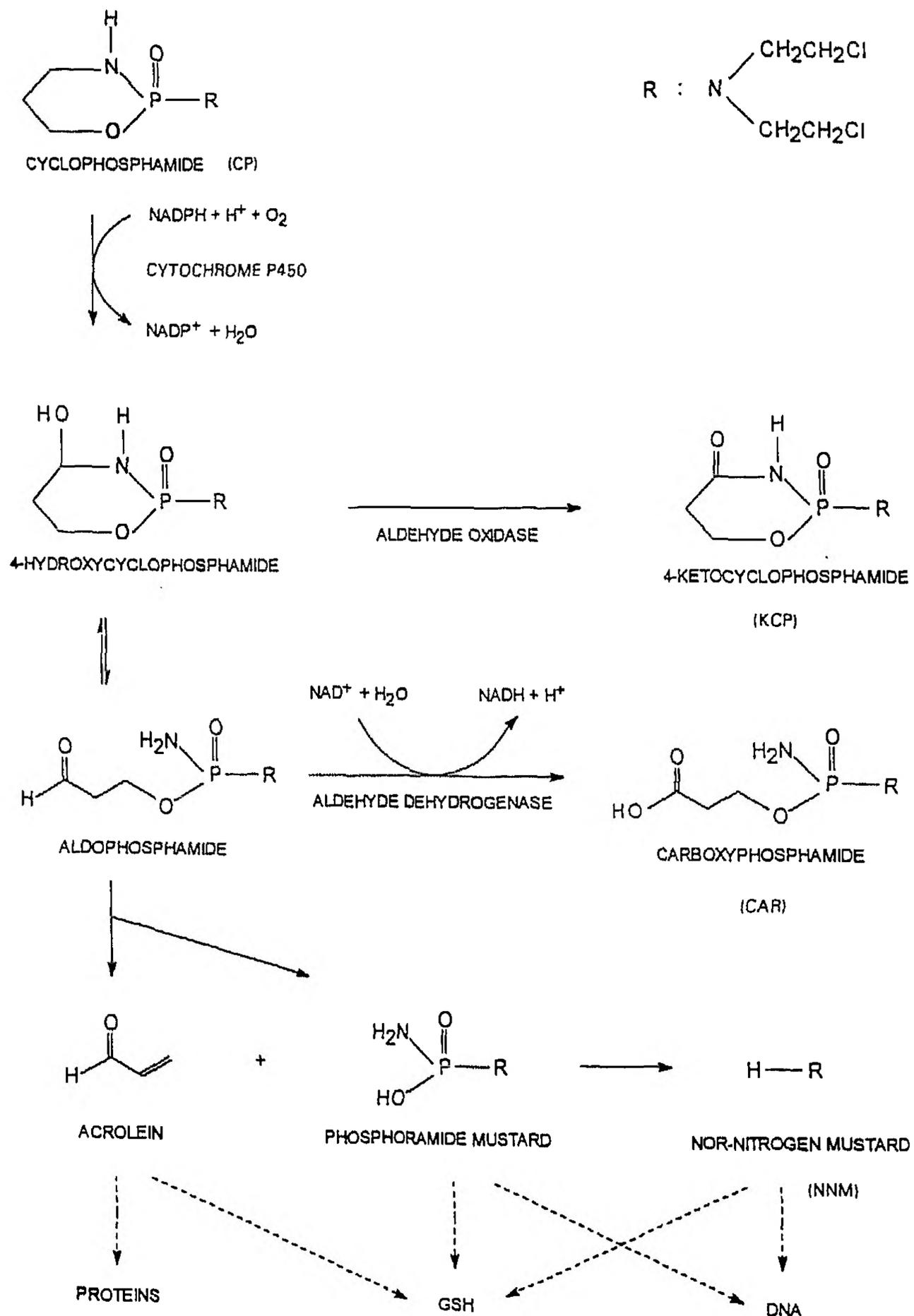

Fig. 1. Abridged scheme of the biotransformation of CP showing only the currently recognized biologically important reactions and metabolites (after Sladek, 1988).

(CAR), respectively. Toxification occurs after β-elimination of acrolein from aldophosphamide to yield phosphoramide mustard which is further converted to nor-nitrogen mustard (NNM). Phosphoramide mustard is responsible for the DNA-alkylating activity resulting in the cytotoxic effect of CP. Acrolein binds covalently to proteins. Most CP(metabolites) are ultimately eliminated by renal excretion.

In several recently published studies we have investigated the exposure of hospital workers to CP (Sessink et al., 1992a,b; 1994a,b; 1995b). It was found that during drug handling the workers were exposed to this compound. Despite the introduced guidelines and protective measures, we were able to detect CP in their urine. It was concluded that uptake of CP due to occupational exposure did happen. The question is to what extent the amounts of CP excreted in urine are a measure for the uptake of the drug. More precisely, what is the relationship between uptake and urinary excretion of CP. Several processes such as the biotransformation, resulting in transformation of the parent compound in metabolites, are involved. Differences in biotransformation are genetically determined but are also influenced by chemical exposure from the environment among which so-called life style factors, that may cause induction or inhibition of enzymes involved in the biotransformation of toxic compounds such as CP (Fournier and Thomas, 1986).

In the present study the in vitro biotransformation of CP was investigated using S9 liver fractions of pretreated and control rats. The biotransformation of CP was studied by the determination of the CP metabolites, nor-nitrogen mustard (NNM), 4-ketyocyclophosphamide (KCP), and carboxyphosphamide (CAR). The effects of the environmental factors, smoking and alcohol, on biotransformation enzymes were mimicked by pretreatment of rats with β-naphthoflavone and ethanol, respectively (Pelkonen et al., 1986; Sesardic et al., 1987). The results were compared with the results obtained from control rats (olive oil and water pretreatment, respectively) and from rats pretreated with Aroclor 1254 and phenobarbital, respectively (positive controls). The influence of sex and supplementation

with NAD and GSH, mimicking a biological variation in NAD and GSH levels in rat and human liver, was also studied. We were especially interested in the balance between toxication and detoxication which is expressed by the toxic/non-toxic metabolites ratio NNM/(KCP+CAR).

Several investigators have studied the in vitro hepatic biotransformation of CP in rats after pretreatment with phenobarbital, Aroclor 1254, β-naphtoflavone and 3-methylcholanthrene (Sladek, 1988; Clarke and Waxman, 1989; LeBlanc and Waxman, 1989). In our study, we were able to detect specific CP metabolites using recently developed gas chromatographic methods after the incubation of CP with S9 liver fractions instead of microsomes or purified cytochrome P450 (iso)enzymes (Sessink et al., 1995a).

The biotransformation of CP was also studied in vivo by determination of unmetabolized CP in urine of rats pretreated with Aroclor 1254. CP was administered by intravenous injection. In addition, dermal application was chosen to mimic the possible dermal uptake as expected during occupational activities of hospital workers (Hirst et al., 1984; Sessink et al., 1994a).

2. Methods

2.1. Animal pretreatment for the determination of CP in urine

Four groups of three male random-bred Wistar rats (Cpb:WU (SPF), body wt about 215 g) were housed individually in stainless-steel metabolism cages and had free access to tap water and RMH food pellets (Hope Farms BV, Woerden, The Netherlands). Urine samples and faeces were collected separately. Two groups of rats were pretreated with Aroclor 1254 (Alltech, Deerfield, IL, USA) in olive oil (i.p., 500 mg/kg body wt, 5 days before CP administration) or olive oil as control (i.p., 2 ml/kg body wt). CP (ASTA-Medica, Frankfurt am Main, Germany) was administered by dermal application or intravenous injection (penis vene) to a group of Aroclor 1254-pretreated rats and to a group of control rats (1 mg/kg body wt). For dermal application, a glycerol suspension was applied on about 2

cm² of the close-shaven skin of the neck. Urine samples were collected on ice water for 24 h. A dose of 1 mg CP/kg body wt is about 10 000 times higher when compared to the occupational exposure of hospital workers to CP (3.6–18 μg CP/day) (Sessink et al., 1995b).

2.2. Animal pretreatment for the in vitro incubations

Six groups of three male and six groups of three female random-bred Wistar rats (Cpb:WU (SPF), body wt about 190 g) were used. The animals were housed per group. They had free access to tap water and RMH food pellets (Hope Farms BV, Woerden, The Netherlands). Groups of male and female rats were pretreated with Aroclor 1254 in olive oil (i.p., 500 mg/kg body wt, 5 days before preparation of the S9 liver fraction), phenobarbital (OPG, Utrecht, The Netherlands) in saline (i.p., male rats: 75 mg/kg body wt, female rats: 50 mg/kg body wt, during 4 days before preparation of the S9 liver fraction), β-naphthoflavone (Janssen, Beerse, Belgium) in olive oil (i.p., 40 mg/kg body wt, during 3 days before preparation of the S9 liver fraction) and olive oil as control (i.p., 2 ml/kg body wt). Groups of ethanol-pretreated male and female rats were obtained by giving tap water containing 15% ethanol for 6 weeks. The corresponding control rats received tap water without ethanol (van de Wiel et al., 1990). The rats were anaesthetized with pentobarbital and finally killed by cervical dislocation.

2.3. Preparation of the S9 liver fractions

After cervical dislocation, the livers were perfused in situ with 0.9% NaCl (w/w), completely removed, weighed and immediately cooled in ice-cold SETH buffer (0.25 M sucrose, 2 mM EDTA and 10 mM Tris–HCl, pH 7.4). Liver homogenates were prepared (20%, w/v) in SETH buffer with a teflon-glass homogenizer. After centrifugation at 9000 × g for 20 min, the floating fat layer was removed. The S9 liver fraction was divided in a number of samples, frozen in liquid N_2 and stored at −80°C until use.

2.4. In vitro incubations

The frozen S9 liver fractions were thawed quickly at 37°C immediately prior to use and

kept on ice. The incubation mixture contained 44 mM MOPS buffer pH 7.4, 2 mM NADP, 5 mM $MgCl_2 \cdot 6H_2O$, 2 mM glucose 6-phosphate, 1 mM EDTA, 4 mM NAD, 5 mM GSH, and an amount of S9 liver fraction based on 0.35 nmol P450. After a preincubation of 5 min at 37°C, the incubation was started by adding 1.5 μmol CP. The final incubation volume was 1 ml. The mixture was mildly shaken during the (pre)incubation at 150 rev./min. The incubation was stopped after 30 min by the addition of 75 μl of a 6 M HCl solution. The reaction mixture was immediately cooled on ice-water. All incubations were carried out double in triplicate. One series in triplicate was used for the determination of CAR and KCP. The other was used for the determination of NNM (Sessink et al., 1995a).

2.5. Enzyme measurements

Total cytochrome P450 was determined from the dithionite-difference spectrum according to Rutten et al. (Rutten et al., 1987). Protein concentrations were measured by the Bradford method using crystalline bovine serum albumin as standard (Bradford, 1976). 7-Ethoxyresorufin-O-deethylase (EROD) and 7-pentoxyresorufin-O-deethylase (PROD) activities were determined according to Burke et al. and aniline-hydroxylase (AH) activity was measured according to Ishidate et al. (Burke et al., 1985; Ishidate et al., 1978). Enzyme and total cytochrome P450 measurements were performed in duplicate. Protein concentrations were measured in triplicate.

2.6. Sample preparation and gas chromatographic analysis of CP in urine

After liquid-liquid extraction and derivatization with trifluoroacetic anhydride, CP was determined with gas chromatography/mass spectrometry (Sessink et al., 1991; 1992b).

2.7. Sample preparation and gas chromatographic analysis of the CP metabolites in the incubation mixture

After liquid-liquid extraction and derivatization, the metabolites are determined with gas chromatography and thermionic specific detection. NNM was determined after derivatization

with benzenesulphonyl chloride. KCP and CAR were determined after derivatization with N,N-dimethylformamide dimethyl acetal (Sessink et al., 1995a).

2.8. Statistical analysis

Two-way analysis of variance (SAS procedure GLM) was used to study influence of pretreatment and administration route for the in vivo experiments. These data were analyzed using Statistical Analysis System version 6.08 (SAS Institute Inc., Cary, NC, USA). Unpaired two-tailed T-tests were used to study influence of pretreatment, sex, and supplementation with NAD and GSH for the in vitro incubations (Tables 2 and 3). Log-transformed data were used. InStat 1.1 was used as the software package. P values below 0.05 were considered to be significant.

3. Results

3.1. Effect of Aroclor 1254 pretreatment and administration route on the urinary excretion of unmetabolized CP

CP was excreted in urine of control rats within 16–20 h after intravenous and dermal administration. After pretreatment with Aroclor 1254, CP excretion was completed at 4–8 h after administration. The cumulative CP excretion as percentage of the dose administered ($\pm$S.D.) was lower in the Aroclor 1254-pretreated rats (intravenous administration: 1.4 ± 0.4; dermal application: 0.6 ± 0.1) compared to the olive oil-pretreated control rats (intravenous administration: 7.6 ± 1.2; dermal application: 5.8 ± 1.1) ($P < 0.0001$). The cumulative CP excretion was also lower after dermal application (Aroclor 1254-pretreated rats: 0.6 ± 0.1; olive oil control rats: 5.8 ± 1.1) compared to intravenous admin-

Table 1
Effect of pretreatment on total cytochrome P450 content, EROD, PROD and AH activities in rat S9 liver fractions[a]

Pretreatment	Total P450		EROD[b]		PROD[c]		AH[d]	
	nmol/mg protein	Fold[e] increase	pmol/min/ mg protein	Fold[e] increase	pmol/min/ mg protein	Fold[e] increase	pmol/min/ mg protein	Fold[e] increase
Male								
Aroclor 1254	0.21	2.1	190	73	9.7	2.5	0.16	2.5
β-Naphthoflavone	0.12	1.2	160	62	5.4	1.4	0.10	1.5
Olive oil control	0.10	1[f]	2.6	1[f]	3.8	1[f]	0.06	1[f]
Phenobarbital	0.24	2.4	29	5.3	15	4.5	0.12	2.5
Ethanol	0.16	1.5	4.5	0.8	3.5	1.0	0.14	2.9
Water control	0.10	1[f]	5.4	1[f]	3.3	1[f]	0.05	1[f]
Female								
Aroclor 1254	0.10	1.9	310	198	16	10	0.08	2.7
β-Naphthoflavone	0.07	1.4	230	145	5.8	3.6	0.05	1.7
Olive oil control	0.05	1[f]	1.6	1[f]	1.6	1[f]	0.03	1[f]
Phenobarbital	0.06	0.6	2.8	0.9	8.1	6.5	0.03	0.6
Ethanol	0.18	1.9	2.6	0.8	1.4	1.1	0.13	2.5
Water control	0.10	1[f]	3.3	1[f]	1.3	1[f]	0.05	1[f]

[a]Values are means of pooled S9 fractions of 3 rats.
[b]EROD, 7-ethoxyresorufin-O-deethylase.
[c]PROD, 7-pentoxyresorufin-O-deethylase.
[d]AH, 4-aniline-hydroxylase.
[e]Induction was compared to olive and water control.
[f]For both male and female rats, olive oil and water were set at 1.

Table 2
Formation of NNM and CAR after incubation of CP with Aroclor 1254- and β-naphthoflavone-pretreated rat S9 liver fractions[a]

	Aroclor 1254		β-Naphthoflavone		Olive oil control	
	♂	♀	♂	♀	♂	♀
Without supplementation with NAD and GSH						
NNM	3300 ± 2000[b*,c***]	38 ± 23	400 ± 240[c**]	17 ± 10	570 ± 350[c***]	8 ± 5
CAR	270 ± 110[b*,c*]	57 ± 23[b*]	53 ± 22[c*]	14 ± 6	74 ± 30[c*]	19 ± 8
NNM + CAR	3600	94	450	30	640	27
NNM/CAR ratio	12.4	0.66	7.6	1.2	7.7	0.40
Supplementation with NAD						
NNM	840 ± 510[c***]	14 ± 9[b***]	80 ± 48[c****,d*]	0[d***]	180 ± 120[c****]	0[d***]
CAR	1200 ± 480[b*,c****,d*]	78 ± 32	160 ± 64[c****,d*]	10 ± 4[b*]	330 ± 140[c***,d*]	36 ± 15
NNM + CAR	2000	92	240	10	520	36
NNM/CAR ratio	0.70	0.18	0.50	0	0.56	0
Supplementation with NAD and GSH						
NNM	50 ± 31[c****,c**]	0[c***]	8 ± 5[c****,c*]	0	14 ± 9[c***,c*]	0
CAR	780 ± 320[b*,c**]	110 ± 44[b*]	120 ± 48[c*]	26 ± 11	190 ± 77[c**]	30 ± 13
NNM + CAR	830	110	130	26	200	30
NNM/CAR ratio	0.06	0	0.06	0	0.07	0

[a]Values of NNM and CAR formation are means ± S.D. of 3 rats (pmol/min/mg protein).
[b]Significantly different from olive oil control pretreated male or female rats.
[c]Significant difference between male and female rats concerning the indicated pretreatment and (*without*) *supplementation with NAD (and GSH)*.
[d]Significantly different from *without supplementation with NAD and GSH* concerning the indicated pretreatment and sex.
[e]Significantly different from *supplementation with NAD* concerning the indicated pretreatment and sex.
*P < 0.05; **P < 0.01; ***P < 0.005; ****P < 0.0005.

istration (Aroclor 1254-pretreated rats: 1.4 ± 0.4; olive oil control rats: 7.6 ± 1.2) ($P = 0.026$). It is concluded that pretreatment with Aroclor 1254 decreased the excretion of unmetabolized CP in urine of rats most likely due to an enhanced biotransformation of CP in vivo. The influence of the administration route was of minor importance indicating almost complete dermal absorption.

3.2. Effect of pretreatment with cytochrome P450 enzyme-inducers on enzyme activities

As was expected, EROD activity was substantially increased in both male and female rats after Aroclor 1254 pretreatment and to a lesser extent after β-naphthoflavone pretreatment in comparison with the olive oil control rats (Table 1). EROD activity was slightly increased in male rats after phenobarbital pretreatment. PROD activity was slightly increased in male and female rats after phenobarbital and Aroclor 1254 pretreatment and in female rats after β-naphtoflavone pretreatment. AH activity was slightly enhanced in male and female rats after Aroclor 1254 and ethanol pretreatment and in male rats after phenobarbital pretreatment.

3.3. Effect of pretreatment with cytochrome P450 enzyme-inducers on the biotransformation of CP

The rates of formation of NNM and CAR after incubation of CP with S9 liver fractions pretreated and control male and female rats, and the influence of supplementation with NAD and GSH are shown in Table 2 (Aroclor 1254, β-naphthoflavone, and olive oil control) and Table 3 (phenobarbital, ethanol, and water control). In

Table 3
Formation of NNM and CAR after incubation of CP with phenobarbital- and ethanol-pretreated rat S9 liver fractions[a]

	Phenobarbital		Ethanol		Water control	
	♂	♀	♂	♀	♂	♀
Without supplementation with NAD and GSH						
NNM	4700 ± 2800[b*,c***]	57 ± 34	610 ± 370[c***]	14 ± 9	390 ± 240[c**]	14 ± 9
CAR	390 ± 160[b*,c*]	94 ± 38[h*]	60 ± 24	26 ± 11	94 ± 38[c*]	18 ± 8
NNM + CAR	5100	150	670	40	490	32
NNM/CAR ratio	11.9	0.61	10.2	0.54	4.2	0.74
Supplementation with NAD						
NNM	2100 ± 1300[b*,c***]	30 ± 18	260 ± 160[c**]	17 ± 10	220 ± 140	60 ± 36
CAR	2400 ± 960[b***,c***,d**]	200 ± 82[b***]	190 ± 76[c*,d*]	42 ± 17	300 ± 120[c***,d*]	20 ± 9
NNM + CAR	4500	230	450	58	520	80
NNM/CAR ratio	0.88	0.15	1.4	0.40	0.74	3.0
Supplementation with NAD and GSH						
NNM	57 ± 35[c***,e***]	3 ± 2[b*,e*]	12 ± 7[c***,e**]	0[e***]	10 ± 7[c**,e**]	0[e***]
CAR	1700 ± 670[b***,e***]	250 ± 98[b***]	150 ± 60[c*]	53 ± 22	210 ± 86[c**]	26 ± 12
NNM + CAR	1700	250	160	53	220	28
NNM/CAR ratio	0.03	0.01	0.08	0	0.05	0

[a]Values of NNM and CAR formation are means ± S.D. of 3 rats (pmol/min/mg protein).
[b]Significantly different from water control pretreated male or female rats.
[c]Significant difference between male and female rats concerning the indicated pretreatment and (*without*) *supplementation with NAD (and GSH)*.
[d]Significantly different from *without supplementation with NAD and GSH* concerning the indicated pretreatment and sex.
[e]Significantly different from *supplementation with NAD* concerning the indicated pretreatment and sex.
$*P < 0.05$; $**P < 0.01$; $***P < 0.005$; $****P < 0.0005$.

none of the incubation mixtures KCP was detected (< 0.5 nmol/ml incubation mixture). Consequently, the formulas for the calculation of the total metabolite formation rate and the toxic/non-toxic metabolites ratio are simplified in NNM + CAR and NNM/CAR, respectively.

3.4. Without supplementation with NAD and GSH

The results show that Aroclor 1254 and phenobarbital pretreatment increased the formation rate of NNM in male rats and the formation rate of CAR in male and female rats compared to their corresponding controls. The formation rate of NNM and CAR was not affected after β-naphthoflavone and ethanol pretreatment. The formation rate of especially NNM and to a lesser extent CAR is higher in male than in female rats

for all pretreatments and controls. Consequently, the total metabolite formation rate NNM + CAR and the NNM/CAR ratio is higher in male than in female rats.

3.5. Supplementation with NAD

Supplementation of the S9 incubation mixtures with NAD decreased the NNM formation rate in β-naphtoflavone-pretreated and in female olive oil-treated control rats. The NNM formation rate was not affected by the other pretreatments and in the water control group. On the contrary, the CAR formation rate was increased in male rats for all pretreatments and their corresponding control groups more or less compensating the decrease of the NNM formation rate. Consequently, the total metabolite formation rate

NNM + CAR was not changed and the NNM/CAR ratio was decreased (except for female water control rats).

In addition, Aroclor 1254 pretreatment increased the formation rate of NNM in female rats and the formation rate of CAR in male rats compared to their corresponding controls. The formation rate of CAR was decreased after β-naphthoflavone pretreatment in female rats. Phenobarbital pretreatment increased the formation rate of NNM in male rats and the formation rate of CAR in male and female rats compared to their corresponding controls. The formation rate of NNM and CAR was not affected after ethanol pretreatment. The formation rate of NNM and CAR is higher in male rats than in female rats for all pretreatments and the control groups (except the NNM formation rate in the water control group).

3.6. Supplementation with NAD and GSH

Supplementation of the S9 incubation mixtures with NAD and GSH decreased the NNM formation rate compared to NAD supplementation alone. The CAR formation rate was not affected. Consequently, the NNM/CAR ratio was decreased. The total metabolite formation rate NNM + CAR was only decreased in the male rats. This is due to the large reduction in the NNM formation rate in the male rats compared to the female rats. These results were not influenced by the pretreatment. Once again, the formation rate of NNM and CAR was higher in male rats than in female rats within each pretreatment.

In addition, Aroclor 1254 and phenobarbital pretreatment increased the formation rate of CAR compared to their corresponding controls. Phenobarbital pretreatment also increased the formation rate of NNM in female rats.

4. Discussion

The cumulative urinary CP excretion (24 h) was significantly decreased in Aroclor 1254-pretreated rats when compared to control rats. An enhanced in vivo biotransformation of CP is

suggested and is confirmed by the results of the incubations in vitro which demonstrate an increase in total CP metabolite formation after pretreatment with Aroclor 1254. These results suggest the influence of external (chemical) factors on CP biotransformation in vivo. The cumulative CP excretion was lower after dermal application compared to intravenous administration. This suggests a higher biotransformation activity after dermal application than intravenous administration. Consequently more metabolites could be formed after dermal application than after intravenous administration. However, it is unknown whether this will result in more toxic or non-toxic metabolites.

Several investigators have studied the in vitro hepatic biotransformation of CP in rats after pretreatment with phenobarbital, Aroclor 1254, β-naphtoflavone and 3-methylcholanthrene (Sladek, 1988; Clarke and Waxman, 1989; LeBlanc and Waxman, 1989). The experiments were carried out with microsomes or purified cytochrome P450 (iso)enzymes. The biotransformation of CP was measured by application of several methods. However, no data about specific CP metabolite formation were presented. It was shown that after phenobarbital pretreatment especially cytochrome P4502B1, contributed to the biotransformation of CP in the rat, whereas cytochromes P4502C6 and 2C11 were responsible for the biotransformation of CP in control rat liver (Clarke and Waxman, 1989; LeBlanc and Waxman, 1989). Recently, it was demonstrated that CP hydroxylation in man was preferentially catalyzed by liver microsomal cytochrome P4502B (Chang et al., 1993). It is suggested that induction of enzymes involved in CP biotransformation results in an increased therapeutic effect since more of the DNA-alkylating metabolite phosphoramide mustard is formed. However, the absence of NAD-linked aldehyde dehydrogenase and aldehyde oxidase in incubations with microsomes and purified P450 systems might overestimate the formation of phosphoramide mustard because metabolites such as CAR and KCP could not be formed. It was our aim to study the effect of enzyme induction and sex on the formation of toxic (NNM)

and non-toxic (CAR and KCP) CP metabolites in rat liver S9 fractions.

In common with other investigators, we also found that the biotransformation of CP was increased after Aroclor 1254 and phenobarbital pretreatment (Sladek, 1988; Clarke and Waxman, 1989; LeBlanc and Waxman, 1989). However, the NNM/CAR ratio was slightly changed after Aroclor 1254 pretreatment but increased after phenobarbital pretreatment. This is possibly caused by a higher increase in CAR formation due to induction of aldehyde dehydrogenase (Lindahl, 1992).

The effects of smoking and alcohol on biotransformation enzymes in the rat were mimicked by pretreatment of the rats with β-naphthoflavone and ethanol, respectively. These so-called life-style factors may cause induction or inhibition of enzymes involved in the biotransformation of toxic compounds such as CP (Fournier and Thomas, 1986). In the present study, no differences in the biotransformation of CP were observed after β-naphthoflavone pretreatment (P4501A1 induction) and after ethanol pretreatment (P4502E1 induction). From these results it could be concluded that pretreatment of rats with β-naphthoflavone and ethanol did not increase CP hydroxylation. Comparable results were found in in vitro experiments with microsomes of human donor livers which showed that P4501A1, 1A2 and 2E1 did not contribute to the hydroxylation of CP (Chang et al., 1993). In addition, we did not find a correlation between EROD activity and NNM, CAR, total metabolite formation and the NNM/CAR ratio in a human liver incubation study (Sessink et al., 1995a). On the contrary, we found a correlation between CAR and total metabolite formation and AH activity while NNM formation and the NNM/CAR ratio were not correlated.

The formation rate of NNM and CAR will differ depending on the biological variation in NAD and GSH levels in rat and human liver. This variation, mimicked by supplementation with NAD necessary for the formation of CAR, increased the formation of this metabolite and decreased the formation of NNM as expected. In a corresponding way, NNM formation was de-

creased after supplementation with GSH. This is explained by conjugation of the electrophiles NNM and phosphoramide mustard.

We have found that the in vitro biotransformation of CP was strongly influenced by sex, and also by supplementation with NAD and GSH. It is concluded that pretreatment with the enzyme-inducers, ethanol and β-naphtoflavone, did not influence the biotransformation of CP in the rat. Hence, it is tempting to conclude that alcohol consumption and smoking, mimicked by pretreatment of rats with ethanol and β-naphtoflavone, respectively, will not affect CP biotransformation in man.

Acknowledgements

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