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INFLUENCE OF LONG-TERM ETHANOL TREATMENT ON *IN VITRO* BIOTRANSFORMATION OF BENZO(*a*)PYRENE IN MICROSOMES OF THE LIVER, LUNG AND SMALL INTESTINE FROM MALE AND FEMALE RATS

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Abstract—The influence of long-term ethanol exposure of rats on the microsomal biotransformation of benzo(*a*)pyrene [B(*a*)P] was studied. Male and female Wistar rats received an increasing amount of ethanol in their drinking water: percentages rose to 15% (w/v) in 3 weeks. The ethanol content was kept at a concentration of 15% for another 3 weeks. Livers, lungs and intestinal epithelial cells of the rats were then isolated and microsomal fractions prepared. In all organs, the metabolite most formed was 3-hydroxy-B(*a*)P. In the liver, males showed significantly higher B(*a*)P hydroxylase activity than females. On the basis of experiments using monoclonal antibodies, a significant part of the B(*a*)P biotransformation in male rat liver microsomes can be attributed to the male specific P4502C11. In the lung and intestine, there were no significant differences between the sexes. In the liver, ethanol treatment significantly decreased the microsomal formation of phenolic metabolites. In microsomes of intestinal epithelial cells, ethanol treatment enhanced the formation of phenols and diols. In conclusion, ethanol consumption by rats in moderate amounts leads to an alteration in the microsomal biotransformation of B(*a*)P. Effects are most prominent in the liver, where the formation of phenols is significantly decreased.

The impact of life style factors on the toxicokinetics of foreign compounds is recognized. The main, non-professional, life style-associated parameters are the use of alcohol and smoking, and drug and dietary habits [1]. Interactions between alcohol intake and the toxicokinetics of polycyclic aromatic hydrocarbons (PAH†) are of interest because PAH are ubiquitous environmental contaminants and components of cigarette smoke. The risk of cancer of the larynx, pharynx and oesophagus is found to be additive or synergistic for people who smoke *and* drink alcohol [2].

Ethanol has a broad spectrum of direct toxic effects [3]. Indirectly, it acts as an inducer of the monooxygenase-linked cytochrome P450 complex [4–7]. Conceivably, the additive or synergistic effect of drinking and smoking on carcinogenesis could be attributed to an enhanced biotransformation of benzo(*a*)pyrene [B(*a*)P] into its reactive intermediates by ethanol-induced P450 monooxygenases. Findings by Seitz *et al.* [8–10] support this idea. They measured enhanced aryl hydrocarbon hydroxylase activity in microsomes of the liver and small intestine of female rats and in intestinal microsomes of male rats that were on an alcohol-containing diet. Also, enhanced biotransformation of PAH in the cheek pouch epithelium (but not in the liver) of Syrian golden hamsters was measured after long-term ethanol treatment [11]. However, other authors found a decrease or no change in the *in vitro* metabolism of B(*a*)P by rat and hamster liver microsomes [12, 13]. The specific ethanol inducible P450 isoenzyme (P4502E1) demonstrated little affinity for PAH [6].

We have described recently the *in vivo* toxicokinetics of B(*a*)P after long-term ethanol treatment.‡ After one i.p. or p.o. dose of B(*a*)P to rats chronically exposed to ethanol via their drinking water, the excretion of 3-hydroxy-B(*a*)P was significantly decreased. The effect of ethanol treatment on B(*a*)P metabolite excretion measured *in vivo* was attributed to changes in absorption, biotransformation and/or excretion.

This paper presents data on the effects of long-term ethanol treatment on the biotransformation of B(*a*)P. Microsomes of the lung, liver and small intestine from male and female rats were used. This allowed for a comparison of possible sex-related differences in B(*a*)P biotransformation in the three organs. Finally, to determine which isoenzyme of P450 was most important in B(*a*)P biotransformation, liver microsomes were incubated with monoclonal antibodies (MAbs) to the P450 isoenzymes, 1A1/2, 2E1, 2B1/2 and 2C11/6, to measure the inhibition of B(*a*)P metabolite formation.

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† Abbreviations: PAH, polycyclic aromatic hydrocarbons; B(*a*)P, benzo(*a*)pyrene; MAb, monoclonal antibody; 3-MC, 3-methylcholanthrene.

‡ van de Wiel JAG, Fijneman PHS, Duijf CMP, Anzion RBM, Theuws JLG and Bos RP, Excretion of benzo(*a*)pyrene and metabolites in urine and feces of rats: influence of route of administration, sex and ethanol pretreatment, submitted.

MATERIALS AND METHODS

Chemicals. Ethanol used for the treatment was Ethanol absolute GR, supplied by Merck (Darmstadt, Germany). B(*a*)P (CAS 50-32-8) was purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). B(*a*)P metabolites were obtained from the NCI Chemical Carcinogen Repository, Midwest Research Institute (Kansas City, MO, U.S.A.). All other chemicals were of analytical grade, obtained from local commercial sources and used without further purification.

Animals. Homebred Cpb:WU (Wistar) rats of both sexes were used. Initial weights varied between 100 and 120 g (females) and 120 and 150 g (males). The age of the rats was about 6 weeks at the beginning of the experiments and 12 weeks at the end. The animals were of Specified Pathogen Free quality. Antibodies against the pneumonia virus of mice and the pin-worm *Syphacia muris* but against no other pathogens were found. The animals were kept in Macrolon type 3 cages on sterilized softwood granules as bedding. The animals were housed three per cage.

The animals were provided with RMH-TM pellets (Hope Farms B.V. Woerden, The Netherlands). The rats had free access to water with an increasing ethanol content reaching up to 15% (w/v) after 6 weeks. Details of the method of alcohol exposure have been described previously [14]. Room temperature was regulated (22 ± 2°); relative humidity varied between 40 and 60%. The animals were exposed to artificial light between 7.00 a.m. and 7.00 p.m.

Preparation of microsomes. Six weeks after the start of the alcohol treatment the rats were anaesthetized with pentobarbital and perfused with ice-cold saline via the ventricle of the heart until the liver and lungs were decoloured. The livers were excised, weighed, collected in ice-cold 0.25 M sucrose, sliced and homogenized in 3 vol. ice-cold 0.25 M sucrose, using a Potter–Elvehjem glass teflon homogenizer. The cell debris and nuclei were removed at a first centrifugal run at 600 *g* (4°). Lungs were also removed, sliced and homogenized in 5 vol. ice-cold 1.15% KCl in 10 mM Tris–HCl/2 mM EDTA and 10% (v/v) glycerol (pH = 7.4). An intestinal segment consisting of the first 60 cm distal to the pylorus was excised, perfused free of intestinal content with an ice-cold isotonic KCl solution containing 0.05 M Tris–HCl buffer (pH = 7.8) and slit open. The upper villous layer of the mucosa was removed by scraping with the edge of a glass slide. Next, it was suspended in 4 mL of Tris–KCl with glycerol (20% v/v) and heparin (3 U/mL). To this suspension, trypsin inhibitor (5 mg/g wet weight of small intestine) was added, according to Stohs *et al.* [15].

From all tissue homogenates mitochondria were removed by centrifugation for 20 min at 9000 *g*. The floating fat layer was removed and the underlying supernatant fraction was decanted and homogenized. The post-mitochondrial fraction was centrifuged at 105,000 *g* for 75 min at 4°. For the liver, the pellet was resuspended in ice-cold 1.15% KCl in 10 mM Tris–HCl (pH = 7.4). The final suspension corresponded to 1 g liver/mL suspension. The microsomal pellet of the lungs was resuspended in 10 mM Tris–HCl (pH = 7.4), according to Boyd *et al.* [16]. The final suspension corresponded to 2 g tissue/per mL. The intestinal microsomes were resuspended to a concentration corresponding to 2 g/mL in 10 mM Tris–HCl containing 1.15% (w/w) KCl, pH = 7.4.

Microsomes of all organs were distributed in small samples, frozen in liquid nitrogen and stored at −80° until use.

Assays. Protein concentrations were determined according to Lowry *et al.* [17] using bovine serum albumin as the standard. The reduced, CO-bound cytochrome P450 difference spectra were determined according to Omura and Sato [18] with modifications according to Rutten *et al.* [19].

The frozen microsomes were thawed quickly at 37° immediately prior to use. B(*a*)P metabolism was assayed by the procedure of Yang *et al.* [20] with modifications. The reaction mixture contained 1.9 mL 0.1 M Sörensen buffer with NADPH (0.72 mM) and $MgCl_2.6H_2O$ (3.75 mM), 60 μL microsomal suspension, and 50 μL B(*a*)P in acetone (100 nmol/mL incubation mixture). After a pre-incubation of 2 min at 37° and shaking at 110 rpm (Gyrotory Water Bath Shaker, New Brunswick Scientific Co., Edison, NJ, U.S.A.), the incubation was started by adding 50 μL of B(*a*)P solution. After 10 (liver) or 20 (lung, intestine) min the incubation was stopped by the addition of 2 mL acetone. B(*a*)P and metabolites were extracted in 4 mL ethyl acetate. After 30 min, 3 mL of the organic layer were removed and evaporated under nitrogen. The residue was dissolved in methanol to a volume of 1.7 mL for the liver and 0.5 mL for the intestine and lung. The samples were kept in vials at −20° until HPLC analysis.

MAb inhibition. MAbs were produced at the US National Cancer Institute, Laboratory of Molecular Carcinogenesis using a modification of the method of Koehler and Milstein [21]. They were tested and characterized as described by Park *et al.* [22, 23] and Ko *et al.* [24]. In the present study four MAbs shown to be specific towards different cytochromes P450s were used as follows: clone 1-7-1 to MC-inducible P4501A1, which cross-reacted with P4501A2; clones 2-66-3 to PB-inducible P4502B1, which cross-reacted with P4502B2; clone 1-91-3 to ethanol-inducible P4502E1 and clone 1-68-11 to P450-2C11, which cross-reacted with P4502C6 [25]. Antibody was added at concentrations of 0, 10, 50 and 100% of the microsomal protein in the incubation mixture. Control MAb (Hy-Hel) was used in the same amounts to determine any non-specific reaction. MAbs were added to microsomes and buffered at room temperature 30 min prior to starting the B(*a*)P metabolism assay at 37° by adding NADPH and substrate.

Reversed-phase HPLC analysis and detection of B(a)P metabolites. Analyses were performed with a Spectra Physics HPLC (SP8800) equipped with two solvent pumps, a solvent programmer and an automatic sampler (SP8775). Using a sample loop, a 20 μL aliquot was injected onto a 150 × 4.6 mm i.d. Nucleosil C18 (10 μm) column. Column

Table 1. Program of Perkin–Elmer LS-4 detector

Time (min)	Excitation (nm)	Emission (nm)	Metabolites detected†
0	280	406	9,10-OH-B(*a*)P
21	265	405	4,5-OH-B(*a*)P/7,8-OH-B(*a*)P
31	265	430	3-OH-B(*a*)P/9-OH-B(*a*)P
41	296	407	B(*a*)P
58	C*	C	

* End of program, back to position at time = 0.

† 3-OH-B(*a*)P, 3-hydroxy-B(*a*)P; 9-OH-B(*a*)P, 9-hydroxy-B(*a*)P; 4,5-OH-B(*a*)P, 4,5-dihydroxy-4,5-dihydro-B(*a*)P; 7,8-OH-B(*a*)P, 7,8-dihydroxy-7,8-dihydro-B(*a*)P; 9,10-OH-B(*a*)P, 9,10-dihydroxy-9,10-dihydro-B(*a*)P.

temperature was 40°, flow 1.0 mL/min. The following solvent programme was 5 min 90% solvent A (60% aqua pure, 40% methanol), a linear gradient to 10% solvent A and 90% solvent B (100% methanol) in 40 min followed by 10 min 90% solvent B.

Fluorescence detection was performed with a Perkin–Elmer LS-4 spectrofluorimeter. The programme for excitation and emission wavelengths during each run is given in Table 1. The retention times of 9,10-dihydroxy-9,10-dihydro-B(*a*)P, 4,5-dihydroxy-4,5-dihydro-B(*a*)P, 7,8-dihydroxy-7,8-dihydro-B(*a*)P and B(*a*)P were 19, 26, 27 and 43 min, respectively. The retention times of 9-hydroxy-B(*a*)P and 3-hydroxy-B(*a*)P were 36.5 and 37.0 ± 0.5 min, respectively. The detection limit for 3-hydroxy-B(*a*)P, 4,5-dihydroxy-4,5-dihydro-B(*a*)P, 7,8-dihydroxy-7,8-dihydro-B(*a*)P, and 9,10-dihydroxy-9,10-dihydro-B(*a*)P was 0.05 nmol/mL injected volume. For 9-hydroxy-B(*a*)P it was 0.01 nmol/mL. Quantification was done by measuring peak heights and comparing these with a standard range incubated and extracted as described for the microsomal incubation samples.

Statistical analysis. Results are presented as mean values ± standard errors of the mean. Statistical analysis was carried out using the Statistical Analysis System package on a VAX6410 minicomputer. The procedure used was general linear model for analysis of variance with unequal cells. A P-value < 0.05 was considered to be of statistical significance.

With the analysis of variance procedure, it was possible to take more than two groups (of rats) and more than one variable (metabolites) into account. The procedure was always applied to four groups (male ethanol, male control, female ethanol, female control). The number of variables (metabolites) tested in one procedure was different for each organ. With the liver samples, each individual metabolite was tested for statistical difference between males and females, and ethanol-treated and control animals. With the lung and intestinal samples, all metabolites were used in one analysis because only a few samples per group were obtained.

RESULTS

Liver

The mean levels of protein and P450 of microsomes from long-term ethanol-treated male and female rats are shown in Table 2. The protein levels were significantly higher in ethanol-treated rats, in both males and females. The levels of P450 were significantly higher in males compared to females and in ethanol-treated rats compared to non-treated rats.

The rates of formation of B(*a*)P metabolites after incubation of B(*a*)P with liver microsomes is shown in Table 3. Quantitatively, the most prominent metabolite formed was 3-hydroxy-B(*a*)P: it accounted for 75% of all metabolites. In males, 9,10-dihydroxy-dihydro-B(*a*)P accounted for 10% of all metabolites and 4,5-dihydroxy-dihydro-B(*a*)P, 7,8-dihydroxy-dihydro-B(*a*)P and 9-hydroxy-B(*a*)P for 5% each of all metabolites measured. In microsomes of female rats 9-hydroxy-B(*a*)P was the second most important metabolite formed (10% of all metabolites measured) and the diols each accounted for 5% of the metabolite total. Male rats showed significantly higher specific B(*a*)P hydroxylase activity than females either per milligram protein or per nmole P450 ($P < 0.001$).

Ethanol-treated rats showed a significantly decreased rate of formation of 3-hydroxy-B(*a*)P and 9-hydroxy-B(*a*)P per milligram protein in comparison with non-treated rats. There is a curious sex-related difference in the mean diol levels. Female rats seem to have a higher diol formation rate after ethanol treatment. On the contrary, male rats have a lower diol formation rate after ethanol treatment. However, these differences were not statistically significant.

Lung

The mean levels of protein and P450 of microsomes from male and female rats after long-term ethanol treatment are shown in Table 4. There were no significant differences in the protein levels nor in the P450 levels (per mg protein) between the sexes and between ethanol-treated and non-treated rats.

The rates of formation of B(*a*)P metabolites in *in vitro* experiments with lung microsomes are shown in Table 5. Only two samples could be measured in each group because of the very small amount of microsomes obtained from one animal. In both males and females, 3-hydroxy-B(*a*)P comprised 70% of all metabolites measured and the diols 9% each.

Table 2. Protein and P450 levels of liver microsomes of male and female rats

Rats	N*	Protein (mg/mL)	P450 (nmol/mg protein)
Male 0% EtOH	6	15.6 ± 0.5	0.77 ± 0.03
Male 15% EtOH	6	18.1 ± 0.9†	1.06 ± 0.06†
Female 0% EtOH	6	13.5 ± 1.2	0.65 ± 0.02
Female 15% EtOH	6	21.3 ± 0.7†	0.74 ± 0.02†

* N, number of rats.
Values are means ± SEM.
† Significant difference between ethanol-treated and non-treated rats $P \leq 0.05$.

Table 3. Rate of formation of B(*a*)P metabolites in liver microsomes of male and female rats

Metabolite	0% EtOH		15% EtOH	
	Male	Female	Male	Female
3-OH-B(*a*)P*	601 ± 47	127 ± 9	425 ± 27	98 ± 4
9-OH-B(*a*)P*	23 ± 2	14 ± 1	20 ± 5	5 ± 0
4,5-OH-B(*a*)P	27 ± 3	5 ± 1	25 ± 4	11 ± 2
7,8-OH-B(*a*)P	42 ± 3	7 ± 1	35 ± 1	8 ± 0
9,10-OH-B(*a*)P†	94 ± 9	9 ± 1	73 ± 9	21 ± 2

N = 6.
Values are nmol/mg protein/min, means ± SEM.
* Significant difference between ethanol-treated and non-treated rats $P \leq 0.05$.
† Significant interaction between ethanol treatment and sex $P \leq 0.05$.
See Table 1 for abbreviations.

Table 4. Protein and P450 levels of lung microsomes of male and female rats

Rats	N*	Protein (mg/mL)	P450 (nmol/mg protein)
Male 0% EtOH	2	9.9 ± 1.3	0.062 ± 0.01
Male 15% EtOH	2	9.3 ± 1.4	0.124 ± 0.03
Female 0% EtOH	2	9.7 ± 0.6	0.093 ± 0.08
Female 15% EtOH	2	8.9 ± 0.9	0.080 ± 0.03

* N, number of samples. Each sample consisted of pooled lung microsomes of 5–6 rats.
Values are means ± SEM.

Table 5. Rate of formation of B(*a*)P metabolites in lung microsomes of male and female rats

Metabolite	0% EtOH		15% EtOH	
	Male	Female	Male	Female
3-OH-B(*a*)P	3.55 ± 1.15	3.34 ± 0.16	2.89 ± 0.25	2.92 ± 0.02
9-OH-B(*a*)P	0.22 ± 0.04	0.13 ± 0.01	0.13 ± 0.01	0.10 ± 0.00
4,5-OH-B(*a*)P	0.39 ± 0.15	0.34 ± 0.02	0.33 ± 0.02	0.35 ± 0.06
7,8-OH-B(*a*)P	0.51 ± 0.20	0.37 ± 0.07	0.47 ± 0.02	0.35 ± 0.00
9,10-OH-B(*a*)P	0.45 ± 0.22	0.37 ± 0.16	0.47 ± 0.01	0.26 ± 0.07

Number of samples = 2. Each sample consisted of pooled lung microsomes of 5–6 rats.
Values are pmol/mg protein/min, means ± SEM.
See Table 1 for abbreviations.

Table 6. Protein and P450 levels of microsomes of the small intestine of male and female rats

Rats	N*	Protein (nmol/mL)	P450 (nmol/mg protein)
Male 0% EtOH	4	16.7 ± 0.9	0.068 ± 0.01
Male 15% EtOH	4	10.1 ± 2.6†	0.085 ± 0.02
Female 0% EtOH	4	11.9 ± 0.5	0.070 ± 0.01
Female 15% EtOH	4	10.8 ± 0.8†	0.053 ± 0.01

* N, number of samples. Each sample consisted of pooled intestinal microsomes of 2–3 rats.

Values are means ± SEM.

† Significant difference between ethanol-treated and non-treated rats $P \leq 0.05$.

Table 7. Rate of formation of B(*a*)P metabolites in microsomes of the small intestine of male and female rats

Metabolite	0% EtOH		15% EtOH	
	Male	Female	Male	Female
3-OH-B(*a*)P	8.39 ± 1.27	6.70 ± 1.08	12.47 ± 1.30	8.00 ± 2.20
9-OH-B(*a*)P	0.95 ± 0.16	0.91 ± 0.12	1.48 ± 0.12	1.06 ± 0.17
4,5-OH-B(*a*)P	0.49 ± 0.14	0.45 ± 0.09	0.70 ± 0.15	0.61 ± 0.10
7,8-OH-B(*a*)P	0.42 ± 0.07	0.20 ± 0.05	0.44 ± 0.23	0.16 ± 0.09
9,10-OH-B(*a*)P	0.09 ± 0.05	0.03 ± 0.03	0.14 ± 0.08	0.03 ± 0.03

Number of samples = 4. Each sample consisted of pooled intestinal microsomes of 2–3 rats.

Values are pmol/mg protein/min, means ± SEM.

Significant difference between ethanol-treated and non-treated rats $P \leq 0.05$ for all metabolites.

See Table 1 for abbreviations.

9-Hydroxy-B(*a*)P was the least important metabolite, about 3% of all metabolites measured. With all metabolites taken together in the analysis of variance, no significant difference between males and females in the formation rate of B(*a*)P metabolites was found. No significant effect of ethanol treatment was observed either.

Intestinal epithelium

The mean levels of protein and P450 of microsomes from male and female rats after long-term ethanol treatment are shown in Table 6. Protein levels were significantly decreased in ethanol-treated compared to non-treated rats. There were no statistically significant differences between the sexes and between ethanol-treated and non-treated rats with respect to the levels of P450 in the microsomes of the small intestine.

In both males and females, 3-hydroxy-B(*a*)P comprised about 85% of all metabolites formed, while the other metabolites amounted to only a few per cent each. Taking all B(*a*)P metabolites into account, B(*a*)P hydroxylation was significantly faster in males than in females ($P = 0.016$) and was significantly enhanced by ethanol treatment ($P = 0.035$). These results are presented in Table 7.

Inhibition of B(a)P metabolism by MAbs

In Table 8, the inhibition of B(*a*)P metabolite formation by MAb 1-68-11 (anti-P4502C11) is shown. The formation of all B(*a*)P metabolites was significantly inhibited. MAbs 1-7-1 (anti-P4501A1), 1-91-3 (anti-P4502E1) and 2-66-3 (anti-P4502B1) did not inhibit or inhibited to only a minor extent the formation of B(*a*)P metabolites (data not shown). The residual activity of B(*a*)P hydroxylase after inhibition with MAb 1-68-11 differed considerably, depending on the metabolite measured. The formation of 9,10-dihydroxy-9,10-dihydro-B(*a*)P was reduced to 17% of the initial value, whereas the formation of 4,5-dihydroxy-4,5-dihydro-B(*a*)P was reduced to only 79%. For 3-hydroxy-B(*a*)P, 4,5-dihydroxy-4,5-dihydro-B(*a*)P and 7,8-dihydroxy-7,8-dihydro-B(*a*)P, there was a significant difference in residual activity between ethanol-treated and non-treated rats when the amount of MAb protein in the incubation mixture was 50% of the amount of microsomal protein. The remaining activity was significantly higher in ethanol-treated rats.

DISCUSSION

Microsomes from the liver, lung and small intestine

Table 8. Inhibition of B(*a*)P metabolite formation by MAbs in microsomes of male Wistar rat liver

Treatment	Metabolite	1-68-11 (P450IIC11/6) (nmol/mg protein/min) 0%	50%	100%
0% EtOH	3-OH-B(*a*)P	1.460 ± 0.203 (100)	0.435 ± 0.142 (46)	0.435 ± 0.142 (31)
	9-OH-B(*a*)P	0.013 ± 0.002 (100)	0.006 ± 0 (42)	0.006 ± 0 (43)
	4,5-OH-B(*a*)P	0.024 ± 0.001 (100)	0.018 ± 0.002 (75)	0.018 ± 0.001 (75)
	7,8-OH-B(*a*)P	0.052 ± 0.004 (100)	0.025 ± 0.002 (49)	0.021 ± 0.002 (40)
	9,10-OH-B(*a*)P	0.128 ± 0.018 (100)	0.022 ± 0.001 (17)	0.022 ± 0 (17)
15% EtOH	3-OH-B(*a*)P	1.240 ± 0.022 (100)	0.627 ± 0.011† (84)	0.472 ± 0.207 (38)
	9-OH-B(*a*)P	0.015 ± 0.001 (100)	0.008 ± 0 (54)	0.007 ± 0.001 (45)
	4,5-OH-B(*a*)P	0.026 ± 0.003 (100)	0.026 ± 0† (87)	0.020 ± 0.006 (79)
	7,8-OH-B(*a*)P	0.054 ± 0.001 (100)	0.042 ± 0.001† (67)	0.023 ± 0.003 (42)
	9,10-OH-B(*a*)P	0.132 ± 0.007 (100)	0.034 ± 0.003 (26)	0.031 ± 0.006 (23)

Values are means of two rat liver samples ± SEM.

In parenthesis the percentage of remaining activity after incubation with the antibody is shown, expressed as (activity with MAb/activity with Hy-Hel) × 100.

† Significant difference between ethanol-treated and non-treated rats $P \leq 0.05$.

See Table 1 for abbreviations.

from male and female rats were used to estimate the effect of ethanol consumption on the biotransformation of B(*a*)P. Quantitatively, the most important metabolite formed in all organs, 3-hydroxy-B(*a*)P, reached up to 70–85% of all metabolites measured. Other authors quoted only 36% in liver microsomes from Sprague–Dawley rats [20] and 10% in intestinal microsomes from Wistar rats [26]. In earlier studies *in vivo*, we found 95% of all excreted metabolites in urine and feces to be 3-hydroxy-B(*a*)P.* This percentage is in agreement with our present results *in vitro*. The second most important metabolite was different in the liver, lung and small intestine. In intestinal microsomes, 9-hydroxy-B(*a*)P was the second most important metabolite. In lung microsomes it was 7,8-dihydroxy-7,8-dihydro-B(*a*)P and in liver microsomes 9,10-dihydroxy-9,10-dihydro-B(*a*)P.

There were large differences in microsomal activity between the three organs. In the liver, B(*a*)P hydroxylase activity was about 1.0 nmol/mg protein/min; in the lung, 0.003 nmol/mg protein/min and in the small intestine, 0.01 nmol/mg protein/min. The different rates of metabolic activity found in these organs are in agreement with the results of other investigators. Benford and Bridges [27] found almost equal values in the liver and small intestine of male Wistar rats. Only in lung microsomes did they find higher activity than we did, about the same as in the small intestine.

In vivo, we measured sex differences with respect to 3-hydroxy-B(*a*)P excretion in the urine and feces: the excretion in males was significantly higher than in female rats. This was in fact reproduced in the present experiments *in vitro*, especially in liver and intestinal microsomes. 9-Hydroxy-B(*a*)P showed no significant sex-related difference *in vivo* nor in liver microsomes. In the organs investigated in this study, males generally showed higher activity than females. Blanck *et al.* [28] showed that oxidative pathways of B(*a*)P biotransformation are controlled by pituitary hormones in a way similar to the metabolism of steroids in rat liver. The results of Yamazoe *et al.* [29] indicate that the concentration of P450 (P4502C11) in liver microsomes from male rats correlates with B(*a*)P hydroxylation activity and that both could be regulated by the serum growth hormone level. Gurtoo and Parker [30] showed a sex-related difference with a factor 6 for arylhydrocarbon hydroxylase activity in liver microsomes of Wistar rats and no sex-related difference in lung microsomes. We found a sex-related difference with a factor of 2–5 for formation of diols and phenols in liver microsomes, no difference in lung microsomes and a small difference in microsomes of the small intestine. Gurtoo and Parker [30] suggest an organ specificity for sex-dependent regulation of microsomal mixed-function oxidase activity. Direct evidence concerning the participation of the male specific isozyme in B(*a*)P biotransformation was provided by Todorovic *et al.* [31]. These authors inhibited microsomal B(*a*)P metabolizing activity using an antibody to P4502C11, a male-specific isozyme. Using the same antibody we were able to inhibit 21–83% of the microsomal B(*a*)P metabolism, depending on the metabolite measured. Like Todorovic *et al.* [31], we also measured the greatest inhibitory effect of MAb 1-68-11 on the formation of 9,10-dihydroxy-9,10-dihydro-B(*a*)P (83%) and the least inhibitory effect on the formation of 4,5-dihydroxy-4,5-dihydro-B(a)P (25%). We also found a considerable inhibition of the formation of the phenolic B(*a*)P metabolites: 54 and 58% for 3-hydroxy-B(*a*)P and 9-hydroxy-B(*a*)P, respectively. Todorovic *et al.* [31] measured only 25%. In Sprague–Dawley rats, sex-specific enzymes as constitutive forms of P450 amount to 20–25% of the total P450

* van de Wiel JAG, Fijneman PHS, Duijf CMP, Anzion RBM, Theuws JLG and Bos RP, Excretion of benzo(*a*)pyrene and metabolites in urine and feces of rats: influence of route of administration, sex and ethanol pretreatment, submitted.

[32, 33]. P4502C11 probably plays an important role in B(*a*)P biotransformation in uninduced as well as in ethanol-treated rats. This is largely different from rats that are treated with the "classic" inducers 3-methylcholanthrene (3-MC) and Aroclor 1254: 3-MC induces P4501A1 that metabolizes B(*a*)P.

The effect of ethanol treatment was different in the three organs. In liver, B(*a*)P biotransformation was significantly decreased for the formation of the phenols 9-hydroxy-B(*a*)P and 3-hydroxy-B(*a*)P. It is known that other P450 inducers like phenobarbital, 3-MC and polychlorinated biphenyls do not affect the sex-specific P450 isozymes or depress them [34]. This also seems to be true for ethanol. When using antibodies to P4502C11/6, the percentage remaining activity of B(*a*)P metabolite formation was significantly higher in ethanol-treated compared to control rats. This confirmed the down-regulating influence of ethanol on sex-specific P450 isozyme activity and consequently on B(*a*)P biotransformation [35]. In the lung also, a decrease in B(*a*)P metabolism was observed, but not a significant one. In the small intestine, a significant increase in the metabolism of B(*a*)P was seen. An increase in B(*a*)P biotransformation in the small intestine was also described by Seitz *et al.* [8]. For the human situation, combined exposure to alcohol and PAH often occurs. It should be realized that ethanol induction could have implications with respect to an altered biologically effective dose of mutagenic/carcinogenic PAH metabolites in several organs.

In conclusion, we found differences between the sexes in the *in vitro* biotransformation of B(*a*)P in agreement with our studies *in vivo*. Males in general have a greater metabolizing capacity for B(*a*)P than females. The influence of ethanol treatment on B(*a*)P biotransformation is different for each organ, but in the liver the formation of phenolic metabolites is significantly decreased. Most probably, in the liver a sex-specific isozyme of P450 (P4502C11) is involved in the biotransformation of B(*a*)P. This isozyme is depressed by ethanol treatment.

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